# Experimental Investigations Into the Role of Dietary Protein on the Control of Energy Intake and Body Mass in Humans

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# Experimental investigations into the role of dietary protein in the control of energy intake and body mass in humans

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# Abstract

Evidence is needed to inform dietary strategies to promote a healthy weight and reduce cardiovascular risk. Dietary protein (P) may influence both energy intake (EI) and energy expenditure (EE). To determine the relationships between appetite control, body mass regulation and protein from different animal and vegetable sources, healthy lean subjects were studied in tightly controlled experiments.

In a 5-way crossover study, 28 subjects aged 19–68 years consumed 2.5 MJ high-protein preload meals (33% energy from protein(P), 34% fat(F), 33% carbohydrate(C)) rich in meat, dairy, nuts and legumes, soy, or control (15%P, 34%F, 51%C), followed by test meals 180 min later. Appetite control mechanisms before, during and for 5 hours after eating, were assessed from analysis of serial blood samples and other physiological measurements. Mean EI was lower after soy (4.1±0.4 MJ) than control (4.6±0.4 MJ), meat (4.7±0.4 MJ), dairy (4.6±0.4 MJ) or nuts/legumes (5.0±0.5 MJ) preloads. Gastric emptying was slower and hunger decreased after soy, and after nuts/legumes, satiation increased and hunger reduced. Blood glucose and insulin excursions were significantly lower after the nuts/legumes and soy meals.

Similar parameters, plus urinary nitrogen, EI and EE were measured in 10 adults during 3 five-day residential experiments. On days 1-3, they ate *ad libitum* diets providing 10%, 15%, or 25% protein, 30% fat and the remainder as carbohydrate. EI was fixed at maintenance requirements on day 4 and EE was measured by calorimeter. EI was lower during the 25%P (8.1±1.0 MJ/d) than the 15%P diet (8.7±1.0 MJ/d) with higher fullness scores, flatter glucose curves and higher pancreatic polypeptide concentration. EE was

similar but fat oxidation was higher on the 25%P diet.

Higher dietary protein promotes energy balance, through decreased energy intake and increased fat oxidation, improves metabolic risk factors, and may be useful to prevent weight gain and related metabolic diseases.

# Declaration

I confirm that the work presented in this thesis is my own. I confirm that information derived from other sources has been indicated as such in the thesis. No part of the material offered has previously been submitted for a degree or other qualification to this or any other university or institution.

Rosemary Megan Hall

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# Abbreviations

| | |
|---|---|
| αMSH | Alpha melanocyte-stimulating hormone |
| °C | Degrees celsius |
| AA | Amino acids |
| ACE | Angiotensin converting enzyme |
| ACTH | Adrenocorticotropic hormone |
| *Ad lib* | *Ad libitum* |
| ADP | Adenosinediphosphate |
| AgRP | Agouti-related peptide |
| ATP | adenosine-5'-triphosphate |
| ATP III | Adult treatment panel III. Third report of the national cholesterol education program expert panel on detection, evaluation, and treatment of high cholesterol in adults |
| AUC | Area under the curve |
| B | Breakfast |
| BMI | Body mass index |
| BMR | Basal metabolic rate |
| Bod Pod | Machine to measure air displacement plethysmography |
| BP | Blood pressure |
| C | Carbohydrate |
| $^{13}C$ | Carbon 13 |
| $CO_2$ | Carbon dioxide |
| CARDIA | Coronary Artery Risk Development in Young Adults study |
| CART | Cocaine-and amphetamine-stimulated transcript peptide |

| | |
|---|---|
| CCK | Cholecystokinin |
| CGMS | Continuous glucose monitoring system |
| CHD | Coronary heart disease |
| CI | Confidence interval |
| CNS | Central nervous system |
| COMA | Committee on Medical Aspects of Food |
| CRF | Wellcome Trust Clinical Research Facility |
| CRP | C-reactive protein |
| CT | Computed tomography |
| d | Day |
| D | Dinner |
| DECODE | Diabetes Epidemiology: Collaborative analysis of Diagnostic criteria in Europe |
| DiOGenes | Diet, Obesity and Genes |
| DIT | Diet-induced thermogenesis |
| DN | Dietary nitrogen |
| DBP | Diastolic blood pressure |
| DPP-IV | dipeptidyl peptidase IV |
| DS | Day Snack |
| DXA | Dual Energy X-Ray absorptiometry |
| EAT-26 | Eating Attitudes Test - 26 item |
| EDTA | ethylenediamine tetra-acetic acid |
| EE | Energy expenditure |
| EEPAA | Energy expenditure for physical activity and arousal |

| | |
|---|---|
| EFSA | European food safety authority |
| EI | Energy intake |
| ELISA | Enzyme-linked immunosorbent assay |
| ES | Evening Snack |
| F | Fat |
| FDA | Food and drug administration (US) |
| FFA | Free fatty acids |
| g | Gram |
| G-6-P | Glucose-6-phosphate |
| G-6-PDH | Glucose-6-phosphate dehydrogenase |
| GEM | ventilated-hood indirect calorimetry system |
| GIP | Glucose-dependent insulinotropic polypeptide |
| GK | Glycerol kinase |
| GLP-1 | Glucagon-like peptide 1 |
| GLP-2 | Glucagon-like peptide 2 |
| GI | Glycaemic index |
| $H_2O_2$ | Hydrogen peroxide |
| HC | High carbohydrate |
| HDL | High-density lipoprotein |
| HF | High fat |
| Hg | mercury |
| HK | Hexokinase |
| HNR | MRC Human Nutrition Research |
| HP | High Protein |

| | |
|---|---|
| hsCRP | High-sensitivity C-reactive protein |
| IAA | Indispensable amino acid |
| ID | Identification |
| IDF | International Diabetes Federation |
| IGF-I | Insulin-like growth factor-I |
| IGF-BP3 | Insulin like growth factor binding protein 3 |
| IgG | Immunoglobulin G |
| kg | Kilogram |
| kJ | Kilojoules |
| L | Lunch, |
| LDL | Low-density lipoprotein |
| LPL | Lipoprotein lipase |
| m | metres |
| MC3 | Melanocortin receptor 3 |
| MC4 | Melanocortin receptor 4 |
| mg | Milligram |
| MJ | Megajoules |
| ml | millilitres |
| mm | millimetres |
| MRI | Magnetic Resonance Imaging |
| MUP | Methyl umbelliferyl phosphate |
| N | Nitrogen |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Nicotinamide adenine dinucleotide (reduced form) |

| | |
|---|---|
| NBL | Nutritional Biochemistry Laboratory |
| NEFA | Non-esterified fatty acids |
| NHANES | National Health and Nutrition Examination Survey |
| NPY | Neuropeptide Y |
| NS | Not significant |
| NST | Nucleus tractus solitarius |
| $O_2$ | Oxygen consumption |
| OXM | Oxyntomodulin |
| P | Protein |
| P:E | Protein to energy ratio |
| PDR | Percentage dose recovered |
| POD | Peroxidase |
| POMC | Pro-opiomelanocortin |
| PP | Pancreatic polypeptide |
| PVN | Paraventricular nucleus |
| PYY | Peptide tyrosine tyrosine |
| REE | Resting energy expenditure |
| Rmax | Maximum rate of label excretion |
| RMR | Resting metabolic rate |
| S | Snack |
| SA | Streptoavidin |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SMR | Sleeping metabolic rate |

| | |
|---|---|
| t | Time |
| t1/2 | Half excretion time |
| TEE | Total energy expenditure |
| TFEQ | Three-Factor Eating Questionnaire |
| tasc | Ascension time |
| tlag | Lag time |
| tlat | Latency time |
| TMB | Tetramethylbenzidine |
| UK | United Kingdom |
| UKPDS | United Kingdom prospective diabetes study |
| UN | Urinary nitrogen |
| USA | United States of America |
| VAS | Visual analogue scale |
| WHO | World Heath Organisation |
| WHR | Waist-hip ratio |
| y | year |
| Y1 | Y1 receptors |
| Y5 | Y5 receptors |

# Chapter 1 Introduction

## 1.1 Energy regulation and body mass

The long-term balance between energy intake and energy expenditure determines body mass (Figure 1.1.1). An individual remains in energy balance if energy intake equals energy expenditure, and therefore body energy stores and body mass remain stable. Energy intake comprises the protein, fat, carbohydrate and alcohol that are consumed as food and drink, while energy output is determined by basal metabolic rate, thermogenesis (induced by diet or cold exposure), physical activity and urinary and faecal losses.

For the most part, body mass is highly regulated through complex homeostatic mechanisms. A change that disturbs energy homeostasis initiates alterations in energy intake or expenditure to return energy balance to baseline (Schwartz *et al.*, 2000). However, an individual's environment modulates eating behaviour which may disrupt these innate control mechanisms. An alteration in energy balance may lead to a positive energy balance, where energy intake exceeds energy expenditure and body energy stores increase, or a negative energy balance, where intake is less than expenditure and energy stores decrease.

**Figure 1.1.1 The energy balance equation**

*Energy balance = Energy intake + Energy expenditure*

### 1.1.1 Regulation of energy intake

Appetite is described as the internal motivation that leads a person to look for food, to select it and to eat it (de Graaf *et al.*, 2004). The act of eating results from the interaction between physiological and psychological factors and adaptation to the environment. Appetite is therefore characterised by motivation to eat (or the sensation of hunger), energy and nutrient intake, and the timing and size of meals and snacks. When satiation develops during a meal, eating is terminated and the satiety that results determines the period until the next meal. During and after a meal, receptors in the wall of the stomach and gastrointestinal tract, and the rate of gastric filling and emptying, interact with receptors in the central nervous system to regulate hunger and satiation. The presence of food and of nutrients absorbed from the gastrointestinal tract stimulates the secretion of hormones and peptides that are involved in satiation, satiety and the development of hunger. Excess energy consumed is stored, in the short term as glycogen and subsequently as triacylglycerols predominantly in adipocytes (but also in muscle and liver). Signals from adipose tissue provide a feedback system to influence hunger and satiety and provide a longer-term control mechanism for energy regulation.

#### *1.1.1.1 The role of satiation and satiety in energy intake*

Total daily energy intake results from a number of eating episodes that differ in size, content and interval and are eaten throughout a 24-hour period, mostly during the daytime. Physiologically, food intake is regulated by two main mechanisms. Satiation develops during an eating episode and terminates eating and therefore determines the amount of food consumed. Satiety is defined as the inhibition of the sensation of hunger after consuming food that prevents further eating in the post-prandial period. As satiety

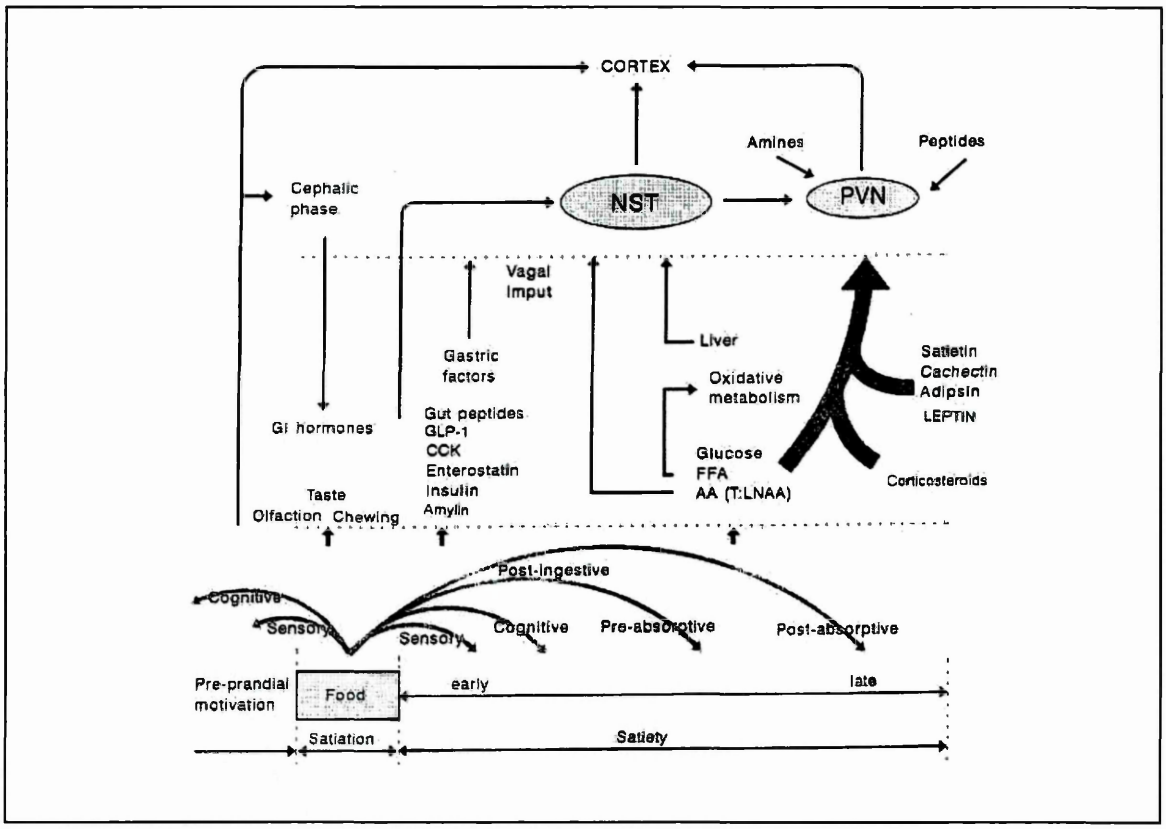
reduces, hunger increases and determines when the next eating episode occurs, though in the modern world habit, social behaviour and other factors complicate this relationship.

Satiety and satiation are modulated by macronutrient composition, energy density, physical structure and sensory qualities of the food recently eaten. Blundell's satiety cascade provides a framework for identifying the physiological mechanisms that mediate satiety and satiation during and after eating (Blundell, 1999). As illustrated in the lower part of Figure 1.1.2 four main processes determine satiety: sensory, cognitive, pre-absorptive and post-absorptive processes.

Sensory signals induced by the senses of sight and smell with contributions from the taste, temperature and texture of foods, determine how palatable a food is and, in the absence of physical or social limitations on its availability, how much is consumed. Anticipating ingestion of food may induce physiological signals before the food has been ingested with secretion of gastric hormones in response to stimulation of the cerebral cortex of the brain, as illustrated in the cephalic phase in the Figure 1.1.2. Cognitive processes to initiate or terminate eating involve an individual's voluntary control of eating, an understanding of the properties of the food consumed, and the social context of the meal. By controlling food availability and the eating environment, the experiments described in this thesis were designed to exclude the effects of cognitive processes and study the physiological factors involved in isolation.

**Figure 1.1.2 Blundell's Satiety Cascade**

The four processes (sensory, cognitive, pre-absorptive and post-absorptive) that regulate satiation and satiety through interactions with the gut, central nervous system and metabolic processes. Abbreviations: NST (nucleus tractus solitarius), PVN (paraventricular nucleus), GLP-1 (glucagon-like peptide 1), CCK (cholecystokinin), FFA (free fatty acids), AA (amino acids). (From Blundell 1999)




Pre-absorptive processes occur in the gastrointestinal tract before nutrients are absorbed. Firstly, gastric filling and distension occurs when food enters the stomach. Vagal mechanoreceptors in the stomach wall are stimulated and contribute to the early phase of satiety (Cummings & Overduin, 2007). Once food has reached the stomach and intestine, cells in the intestinal wall are stimulated to secrete hormones that reduce food intake (such as cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1) and peptide tyrosine tyrosine (PYY), and pancreatic polypeptide (PP) from the pancreas) and inhibit the

secretion of hormones that stimulate eating (such as ghrelin). Glucose, fatty acids, and amino acids released by the digestion of food stimulate vagal chemoreceptors in the intestinal wall that additionally promote satiety.

Post-absorptive processes that regulate satiety occur mainly from the metabolism of macronutrients. The rise in blood glucose after carbohydrate digestion and absorption stimulates satiety while a fall in blood glucose stimulates hunger (de Graaf *et al.*, 2004). Fatty acids, resulting from the digestion of fat, and amino acids, produced with the digestion of protein, appear to regulate hunger indirectly. When these nutrients enter the bloodstream secretion of hormones such as leptin and insulin increases, and have both immediate and long-term effects on satiety regulation (Badman & Flier, 2005).

#### *1.1.1.2 The role of the central nervous system in energy intake*

The central nervous system (CNS), responds to and determines immediate and long-term energy balance by integrating neural, endocrine and metabolic signals to produce behavioural, autonomic and endocrine responses (Spiegelman & Flier, 2001) (Badman & Flier, 2005). This complex system is illustrated in Figure 1.1.3.

One of the first signals to reach the CNS is from the nutrient and stretch receptors in the gastrointestinal tract during the pre-absorptive phase of satiety. Signals are transmitted through the vagus nerve to the nucleus tractus solitarius (see Figure 1.1.2) in the hindbrain and influence meal timing and size and therefore short-term energy balance (Badman & Flier, 2005).

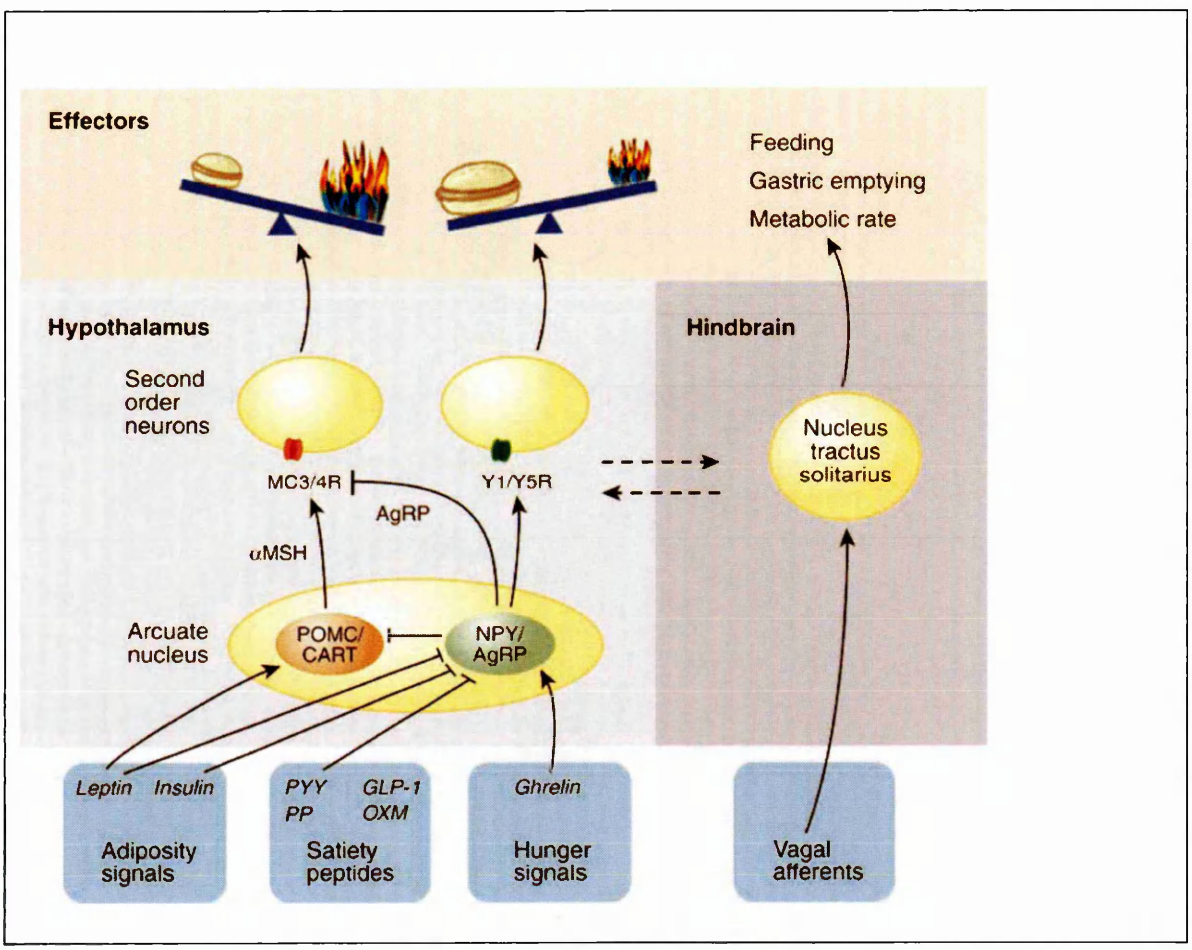
In the subsequent pre-absorptive and post-absorptive phases following a meal the

hypothalamus has a central role in energy balance. The two main areas in the hypothalamus involved in energy regulation are the arcuate nucleus and the paraventricular nucleus. Stimulation of pathways in the arcuate nucleus inhibits eating, while stimulation of pathways in the paraventricular nucleus increase eating. In the rat, inactivating the pathways that inhibit eating by experimental lesions in the ventromedial and arcuate nuclei of the hypothalamus produce hyperphagia and eventually obesity. Conversely, lesions in the paraventricular nucleus in the lateral hypothalamus (where pathways increase feeding) cause aphagia and weight loss (Elmquist *et al.*, 1999).

Neurons in the arcuate nucleus secrete specific peptides which act on second order neurons in the paraventricular nucleus to suppress or stimulate eating. Appetite inhibiting neurons in the arcuate nucleus contain pro-opoimelanocortin (POMC) peptides (such as α melanocyte stimulating hormone (αMSH)) and cocaine-and amphetamine-stimulated transcript peptide (CART) (Figure 1.1.3). αMSH acts on melanocortin 3 and melanocortin 4 receptors to suppress feeding activity. Appetite stimulating neurons are also present in the arcuate nucleus. They secrete neuropeptide Y (NPY) and agouti-related peptide (AgRP). NPY acts on Y1 and Y5 receptors and AgRP inhibits melanocortin receptor activity in the paraventricular nucleus to increase food intake. NPY also inhibits the effect of POMC neurons to further stimulate eating (Badman & Flier, 2005).

**Figure 1.1.3 Central nervous system regulation of satiety**

(Badman and Flier 2005). A representation of the effect of peripheral signals and their receptors that act on the hypothalamus and hindbrain to modulate eating behaviour. POMC (pro-opiomelanocortin), αMSH (α melanocyte-stimulating hormone), MC3 and MC4 (melanocortin receptors), CART (cocaine-and amphetamine-stimulated transcript peptide), NPY (neuropeptide Y), Y1 and Y5 (Y receptors), AgRP (agouti-related peptide), PYY (peptide YY), PP (pancreatic polypeptide), GLP-1 (glucagon-like peptide 1), OXM (oxyntomodulin), →, direct stimulatory; ⊣ direct inhibitory; ⇢, indirect pathways. R means receptor.



Peripheral signals, primarily from the gut and adipose tissue, are able to cross the blood brain barrier to interact with the neurons in the hypothalamus. Gut hormones, particularly PYY, inhibit the action of NPY thereby reducing feeding. Adiposity signals, leptin and insulin, also inhibit NPY to prevent the stimulation of eating, while leptin also acts directly on the POMC neurons to inhibit food intake. The gut hormone ghrelin, is the main promoter of eating by stimulating NPY-secreting neurons, and therefore acts as a signal to initiate food intake.

### *1.1.1.3 The role of the gastrointestinal tract in energy intake*

#### 1.1.1.3.1 Gastric filling and emptying

The sense organs that detect gastric filling, and the rate of gastric emptying are important in the initial regulation of eating and the subsequent release of the hormones that influence satiety and energy balance. The stomach wall contains a large number of mechanoreceptors which sense the stretch arising from increased volume of the stomach after food has been ingested. Signals from these receptors are relayed to the brain by vagal and spinal sensory nerves and contribute to satiation during a meal (Cummings & Overduin, 2007). The initial gastric distension during a meal may also contribute to the release of gastrointestinal hormones in the upper and lower intestines which, as well as coordinating digestion, influence meal satiation and post-prandial satiety.

The rate of gastric emptying during and after a meal contributes to appetite and food intake. The volume and weight of a meal, its physical form (liquid or solid), composition and energy content all influence gastric emptying (Jackson *et al.*, 2004). Delayed gastric

emptying enhances both short-term satiation and between meal satiety. When food remains in the stomach for a longer period the activated stretch receptors prolong the stimulus to the brain which inhibits further eating. Additionally a slower rate of gastric emptying prolongs post-prandial secretion of gastrointestinal hormones, increasing post-meal satiety. Intestinal signals further enhance the satiating effect by delaying gastric emptying (Cummings & Overduin, 2007).

#### 1.1.1.3.2 Gastrointestinal hormones

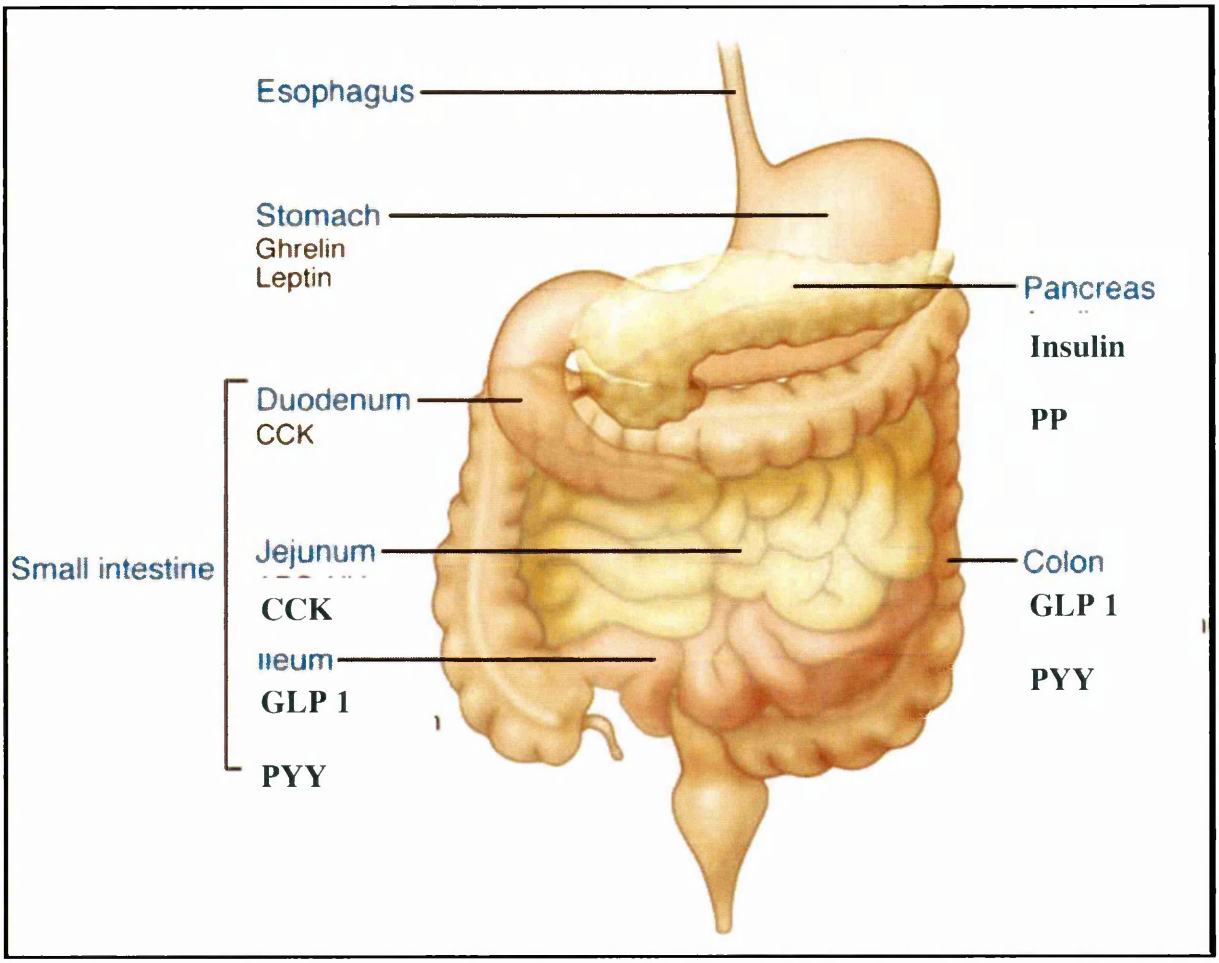
The gut-brain axis is an important link between food intake and appetite regulation. Satiety and body energy store signals, processed in the brain, alter food intake in a manner intended to maintain energy homeostasis. Hormones are secreted from enteroendocrine cells along the entire length of the gastrointestinal tract and, by interacting with neurons bearing appropriate receptors in the brain, produce signals which act as meal initiators and terminators, as summarized in Figure 1.1.4 (Chaudhri *et al.*, 2008).

#### 1.1.1.3.3 Cholecystokinin

Cholecystokinin (CCK) is produced by enteroendocrine I cells in the duodenal and jejunal mucosa. It is rapidly released in response to nutrients in the gut, particularly lipids and proteins (Wynne *et al.*, 2004). CCK acts via the vagal nerve to coordinate post-prandial gall bladder contraction and pancreatic secretion and controls gastric emptying and gut motility (Badman & Flier, 2005). Experimental CCK administration delays gastric emptying which may be the main contributor to the effect of CCK on satiety.

Infusions of CCK reduce meal size and duration in humans, and when the action of CCK is experimentally blocked in rodents weight gain occurs (Wynne *et al.*, 2004).

**Figure 1.1.4 The gastrointestinal tract indicating the locations where gut hormones are released.**




1.1.1.3.4 Glucagon-like peptide 1

Glucagon-like peptide 1 (GLP-1) is cleaved from preproglucagon (Holst, 1999). It is released from L-cells in the distal small intestine and colon in response to nutrients, at levels in proportion to energy intake (Wynne *et al.*, 2004) and inhibits appetite to reduce food intake (Holst, 1999). GLP-1 is an incretin hormone (a hormone that stimulates

insulin secretion) and regulates glucose-dependent insulin release, inhibits glucagon secretion and increases pancreatic ß-cell growth (Karhunen *et al.*, 2008). Circulating levels rise after food intake, particularly after a glucose load. An interesting feature of GLP-1 is that levels increase shortly after a meal, prior to nutrient contact with the distal intestine. GLP-1 secretion is also stimulated via indirect neurohormonal mechanisms in response to nutrients, particularly carbohydrates and fats, in the duodenum. Additionally, GLP-1 plays an important role in the 'ileal brake', a mechanism regulating the flow of nutrients from the stomach into the small intestine, to slow gastric motility and gastric emptying, thereby increasing satiety shortly after a meal (Cummings & Overduin, 2007).

Exogenous GLP-1 reduces energy intake in a dose-dependent manner (Verdich *et al.*, 2001) and stimulates glucose-induced insulin secretion, reduces glucagon levels and slows gastric motility (Kreymann *et al.*, 1987). In type 2 diabetes, GLP-1 infusions normalise glucose and enhance weight loss with a possible improvement in insulin sensitivity and pancreatic beta cell function (Heine *et al.*, 2005). The limiting factor in the therapeutic use of GLP-1, or its receptor agonists, is that it is rapidly inactivated by the enzyme dipeptidyl peptidase IV (DPP-IV). However with the development of long-acting GLP-1 analogues and DPP-IV inhibitors these agents are now in widespread use for the treatment of type 2 diabetes, and some GLP-1 analogues are being tested as treatments for obesity.

#### 1.1.1.3.5 Peptide YY

Peptide YY (PYY), a 36-amino acid peptide, is secreted from L cells in the lower intestine, many of which co-express GLP-1 (Adrian *et al.*, 1985). It is secreted post-

prandially in proportion to the energy load, particularly in response to lipid ingestion. As with GLP-1, PYY secretion occurs soon after a meal in response to neural stimulation in the duodenum and subsequently by direct nutrient stimulation in the lower intestine. PYY, along with NPY, is part of the pancreatic polypeptide-fold protein family which interact with neuropeptide Y receptors (of which there are 5 known receptors labelled Y1 to Y5) and couple to inhibitory G proteins (Cummings & Overduin, 2007). PYY exists in an intact form, PYY(1-36) and as PYY(3-36) where the N-terminal tyrosine-proline dipeptide has been cleaved by DPPIV (Grandt *et al.*, 1994). PYY(3-36) binds to neuropeptide Y2 receptors in the hypothalamus where it inhibits NPY neurons, reduces the NPY-induced inhibition of POMC neurons, and stimulates signals that decrease food intake. PYY also delays gastric emptying.

The intact PYY(1-36) peptide does not appear to modulate appetite or energy intake (Sloth *et al.*, 2006) but infusions of the cleaved form, PYY(3-36), to match post-prandial levels, decrease food intake and body weight in rodents and reduce appetite and energy intake in healthy human subjects (Batterham *et al.*, 2002). PYY(3-36) has been shown to increase diet-induced thermogenesis, post-prandial blood insulin and glucose concentrations and free fatty acid secretion, possibly due to increased lipolysis (Sloth *et al.*, 2006). PYY may be the key mediator of the satiating effects of protein (Batterham *et al.*, 2006).

#### 1.1.1.3.6 Pancreatic polypeptide

Pancreatic polypeptide (PP) belongs to the same PP-fold peptide family that includes neuropeptide Y (NPY) and PYY. PP is secreted primarily by the endocrine cells of the

pancreas with some secretion in the exocrine pancreas, colon and rectum (Karhunen *et al.*, 2008). Secretion of PP is controlled by the parasympathetic nervous system and is regulated by food intake and by an intrinsic circadian rhythm (Stanley *et al.*, 2004). Food intake stimulates a biphasic release of PP in proportion to energy intake, and levels may remain elevated for up to 6 hours post-prandially (Adrian *et al.*, 1976). An increase in PP concentration has also been observed following adrenergic stimulation due to exercise or hypoglycaemia. In addition, sham feeding or gastric distension by water ingestion stimulate PP secretion (Witteman *et al.*, 1994), (Karhunen *et al.*, 2008).

Peripherally administered physiological doses of PP in mice produce a rapid, prolonged reduction in food intake, acting within 20 minutes and persisting for 24 h (Asakawa *et al.*, 2006) (Asakawa *et al.*, 2003). Additionally, transgenic mice overexpressing PP are lean, have reduced food intake and reduced gastric emptying (Ueno *et al.*, 1999). There appears to be a similar effect in humans. PP infusions provided to 10 healthy volunteers reduced appetite and energy intake at a lunch two hours post-infusion, with a sustained effect that reduced the 24 h energy intake by 25% (Batterham *et al.*, 2003).

#### 1.1.1.3.7 Ghrelin

The orexigenic hormone ghrelin is an acylated 28-amino acid agonist of the growth hormone secretagogue receptor formed by the cleavage of preproghrelin (Kojima *et al.*, 1999). It is released primarily from cells in the fundus of the stomach and the proximal small intestine in response to circulating and nutritional signals, in proportion to energy intake, and activates the orexigenic neurons expressing NPY in the hypothalamus. (Wynne *et al.*, 2004). Plasma concentrations of ghrelin rise before a meal and fall to a

trough within one hour after eating (Cummings *et al.*, 2001), suggesting a role in initiation of eating. Ghrelin further stimulates eating by increasing gastric motility and decreasing insulin secretion.

Exogenous ghrelin stimulates food intake in rats (Tschop *et al.*, 2000) (Nakazato *et al.*, 2001) and humans (Wren *et al.*, 2001) and reduces the time between meals to further increase energy intake. Ghrelin levels are lower in obesity (Tschop *et al.*, 2001) and rise after weight loss (Cummings *et al.*, 2002) possibly acting as an adaptive mechanism to restrict diet-induced weight loss. Cummings *et al* (Cummings *et al.*, 2002) found ghrelin levels were 77% lower in patients after gastric bypass surgery (when the gastric fundus is by-passed) than in lean control subjects, suggesting that lack of ghrelin secretion after surgery contributes to weight loss. Reports of the long-term outcomes of gastric bypass surgery have not consistently shown a decrease in ghrelin, possibly due to the type of operation performed and whether ghrelin levels were measured during the weight loss phase or at a stable weight (Williams & Cummings, 2005). It is apparent that ghrelin has a role in energy balance though its exact association with dietary intake and weight regulation requires clarification.

#### *1.1.1.4 The role of adiposity signals in energy intake*

Insulin is released from the pancreatic beta cells in response to blood glucose levels and contributes to short-term control of food intake and long-term energy regulation. It acts through the insulin receptor to remove glucose from the blood into muscle and other tissues and suppress glucose secretion from the liver.

Insulin receptors are also expressed in the hypothalamus where the presence of insulin reduces food intake and increases energy expenditure (Plum *et al.*, 2005). Insulin circulates in the blood in proportion to body fat (Schwartz *et al.*, 2000) and enters the brain in proportion to plasma levels. The presence and action of insulin in the central nervous system is therefore dependent on adiposity and provides a key feedback signal in body weight regulation (Woods *et al.*, 2006). With weight gain, insulin sensitivity is reduced, necessitating increased insulin release to maintain glucose homeostasis. More insulin reaches the hypothalamus to signal a reduction in food intake and limit further weight gain. Conversely, in uncontrolled Type 1 diabetes mellitus, a deficiency of insulin causes hyperphagia. However, as insulin is required for fat deposition (and leptin synthesis in adipocytes) weight gain does not occur in a state of insulin deficiency, as excess glucose is secreted in the urine.

Circulating levels of leptin, a hormone secreted predominantly by adipocytes, are roughly in proportion to body fat, but there is much unexplained inter-individual variation (Considine *et al.*, 1996). Like insulin, leptin enters the central nervous system to interact with leptin receptors in the hypothalamus and regulate food intake and energy expenditure (Friedman & Halaas, 1998; Schwartz *et al.*, 2000). Leptin is the dominant adiposity signal in the central nervous system and has an important role in energy homeostasis (Badman & Flier, 2005). Centrally administered leptin reduces food intake and increases energy expenditure leading to loss of adipose tissue (Halaas *et al.*, 1995) (Friedman & Halaas, 1998). Leptin levels are reduced with food restriction and starvation which leads to an increase in appetite and a reduction in energy expenditure. During starvation or in the absence of leptin, reproduction and growth are inhibited, further

reducing energy expenditure (Spiegelman & Flier, 2001). Examining leptin deficient mice and the small number of leptin-deficient humans illustrates the function of leptin most effectively. Individuals with a mutation in the leptin gene, which disrupts the production of leptin, have very low levels of active leptin and have continual hyperphagia from a young age which quickly leads to severe obesity (Zhang *et al.*, 1994; Montague *et al.*, 1997) that is corrected by administering leptin (Halaas *et al.*, 1995; Farooqi *et al.*, 1999). In obese humans without leptin deficiency, leptin levels continue to rise in proportion to body fat, but appetite is not suppressed and fat mass increases (Considine *et al.*, 1996). This situation suggests a reduction in leptin sensitivity from defective transport to the CNS or impaired intracellular signalling (Badman & Flier, 2005).

The stomach wall produces small amounts of leptin and, while its role is unclear, gastric leptin may also play a role in short-term energy regulation (Meier & Gressner, 2004). Like ghrelin, the release of gastric leptin may occur in response to post-prandial insulin levels (Obici *et al.*, 2001). Intraperitoneal injection of leptin in rats reduces meal size and terminates eating (French & Castiglione, 2002). When leptin was replaced in a patient with congenital leptin deficiency, energy intake reduced significantly within seven days, suggesting an acute affect of leptin on energy intake despite the presence of similar adipose tissue mass (Farooqi *et al.*, 1999).

### 1.1.2 Regulation of energy expenditure

Energy expenditure is measured as the amount of heat released by the body (Das & Roberts, 2001). There are three main components of energy expenditure: basal metabolic rate (BMR) or resting metabolic rate (RMR), thermogenesis, and energy expenditure for

physical activity and arousal (EEPAA). Together these components reflect 24-hour total energy expenditure (TEE).

BMR is the rate of energy expenditure while lying in bed at rest, 12-14 hours after the last meal. It can account for 60-70% of TEE depending on an individual's level of physical activity. BMR is correlated with fat-free mass so increasing muscle mass increases BMR and may provide a mechanism to increase TEE. It is noteworthy that BMR is higher in the obese because of an increase in fat-free mass as well as fat mass (Prentice *et al.*, 1986). This finding negated earlier theories that obesity is caused by a low BMR (Schutz *et al.*, 1984). BMR decreases with age (Roberts *et al.*, 1995) and is lower in women (Ferraro *et al.*, 1992), with small fluctuations in BMR throughout the menstrual cycle (Davidsen *et al.*, 2007).

Thermogenesis is the process of heat production and occurs after eating, exercise, or in response to stimuli such as cold. In humans, the largest component is the energy expended with ingestion, absorption, transport, storage and utilization of food, diet-induced thermogenesis (DIT) (Das & Roberts, 2001). It is composed of energy used in the processing and storage of ingested nutrients, and increased activity of the sympathetic nervous system from the sensory and metabolic stimulation of food. DIT is proportional to the size of the meal consumed and accounts for 7-13% of energy expenditure (Schutz *et al.*, 1984).

EEPAA is the energy expenditure which occurs during physical activity and arousal. As a proportion of TEE it typically varies from 30% in sedentary individuals up to 70% in

highly active individuals. Measuring EEPAA is technically more difficult than the other components of TEE as there is a large variability in energy expended during occupational activity, leisure activity, and residual arousal and non-specific activity such as fidgeting. EEPAA is however a modifiable component of EE, whereas the other components reflect basic physiological processes.

Recent evidence has identified the presence of brown adipose tissue in humans which persists beyond the newborn period and may be important in cold-induced thermogenesis and potentially in the development of obesity (Virtanen *et al.*, 2009). Nuclear medicine scans, to investigate tumour metastasis, demonstrated brown adipose tissue in adult humans, particularly in cold conditions (Nedergaard *et al.*, 2007). In 10 lean and 14 obese subjects, brown adipose tissue was found to be present during cold exposure in all but one subject, but not under thermoneutral conditions (van Marken Lichtenbelt *et al.*, 2009). Tissue activity was reduced in overweight and obese men and was associated with a decrease in basal metabolic rate. An inverse association between the amount of active brown adipose tissue and body mass suggests a potential role in adult human metabolism that is currently being investigated (Cypess *et al.*, 2009).

24 h TEE is a reflection of net EE and may be measured either under controlled conditions in a calorimeter, or in free-living individuals using doubly-labelled water. These techniques and others that have been used to measure the components of TEE are discussed in Chapter 2.

## 1.2 Consequences of poor energy regulation

The physiological factors discussed above are designed to tightly control energy balance and maintain body mass in the long term. Remarkably, this balance is usually achieved and body mass remains stable. However, a sustained small imbalance between energy intake and energy expenditure can produce a substantial change in body mass. For every 1% (approximately 100 kJ/day) increase in energy intake relative to energy expenditure body mass increases by approximately 15kg/decade (Das & Roberts, 2001). Imbalances in energy regulation lead to the most common public health nutrition problems today; overnutrition and undernutrition. In the economically developed world, and increasingly in low and middle-income countries, overnutrition, leading to obesity, is a considerable and increasing problem. The diseases associated with obesity are a major cause of morbidity and premature mortality, with high health care costs (Seidell, 2000). This thesis focuses exclusively on the consequences of positive energy balance and investigates the potential for changes in diet composition to maintain balance and prevent the development of obesity.

### 1.2.1 Obesity

In England and Wales, mean Body Mass Index (BMI) has increased steadily during the last 50 years and obesity (BMI>30 $kg/m^2$) has trebled since the 1980s. In 2008 24.5% of UK adults were obese and 61.4% overweight (BMI>25 $kg/m^2$) (NHS, 2009). Similar increases in the prevalence of obesity have been seen in Europe (Seidell, 2000). The United States National Health and Nutrition Examination Survey (NHANES) identified 33.8% of adults as obese and 68% as overweight in 2007-2008 (Flegal *et al.*). With one billion people worldwide overweight and more than 300 million obese the World Health

Organisation has described the problem as a "worldwide epidemic" (WHO, 2003). Of increasing concern is the development of obesity at an earlier age, particularly the increasing worldwide prevalence of obesity in children (Lobstein *et al.*, 2004).

Overweight or obesity occurs when energy intake exceeds energy output and excess energy is stored as triacylglycerol in adipose tissue. Although physiological regulators maintain energy balance, the increasing prevalence of obesity suggests that they are readily overwhelmed by environmental factors. As the prevalence of overweight and obesity increases, the need to identify environmental influences, such as dietary factors, to prevent weight gain or promote weight loss has become increasingly important.

#### *1.2.1.1 Definition and Measurement*

To define obesity, the World Health Organisation recommends a classification system based on Body Mass Index (BMI) (WHO, 2000), calculated as body mass in kilograms divided by height in metres, squared (BMI = $kg/m^2$). An adult with a BMI of 18.5 – 24.9 $kg/m^2$ is within the normal range, 25.0 to 29.9 $kg/m^2$ is overweight and over 30 $kg/m^2$ is classified as obese. The classification relates to the progressive increase in risk of morbidity and mortality from diseases such as type 2 diabetes, hypertension and coronary artery disease associated with an elevated BMI (WHO, 2000; Yusuf *et al.*, 2005).

It is recognised that the measure of BMI is not a reliable measure of fatness for individuals. It is a useful surveillance tool to estimate the prevalence of obesity but does not allow for differences in body composition or adipose tissue distribution between different races or by gender, and may not indicate the same health risks for different

individuals and populations. Awareness of a group of 'metabolically normal' obese, in whom BMI is elevated but metabolic profile and risk factors for metabolic disease remain normal, has emphasised the need to identify alternative methods for assessing overweight (Karelis *et al.*, 2004).

The presence of excess adipose tissue is an indicator of risk for metabolic disease especially if fat is stored intra-abdominally. Measurement of waist circumference is a simple method of estimating abdominal adipose tissue. The WHO defined limits for waist circumference, which indicate a substantially increased risk of metabolic complications, are 102 cm for men and 88 cm for women (WHO, 2000). Higher values have been associated with hyperlipidaemia, impaired glucose metabolism, hypertension, endothelial dysfunction, elevated inflammatory profile and prothrombotic susceptibility (Despres *et al.*, 2001) (Yusuf *et al.*, 2005). The International Diabetes Federation has redefined central obesity as $\geq$ 94 cm for men and $\geq$ 80 cm for women and introduced ethnic-specific waist circumference cut-offs to recognise the increased risk for metabolic disorders with enlarged abdominal fat, and therefore higher waist circumference, and the increasing susceptibility of some ethnic groups to the deposition of abdominal adipose tissue (Alberti *et al.*, 2005) (IDF, 2005).

A further marker of adiposity, an increased waist-to-hip ratio (waist circumference as a fraction of hip circumference), is defined as >1.0 in men and >0.85 in women (WHO, 2000). Despite debate over whether this ratio is as reliable an indicator of abdominal adiposity as waist circumference alone (Despres *et al.*, 2001) a high waist-to-hip ratio is associated with obesity-related diseases and increasing cardiovascular risk (Yusuf *et al.*,

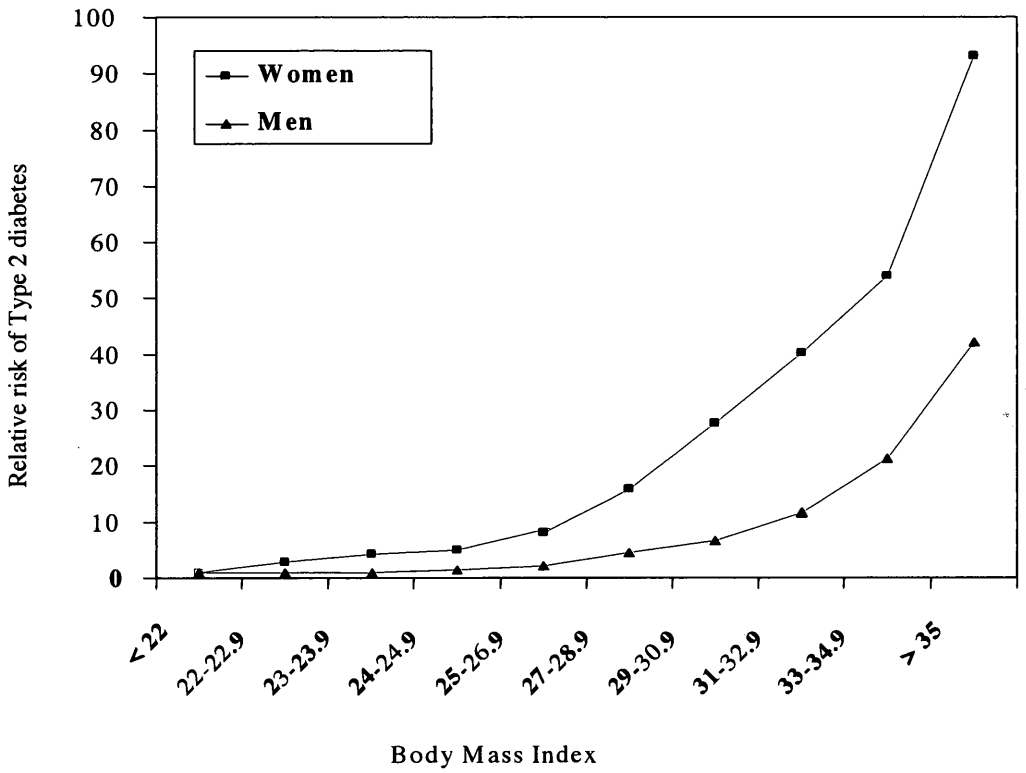
2005). Including a measure of hip circumference may provide information about gluteal muscle mass and estimate total skeletal muscle mass. In support of measuring both waist and hip circumference, Heitmann *et al* (2004) demonstrated that a large hip circumference, relative to waist and BMI, protects women against cardiovascular disease and coronary heart disease (Heitmann *et al.*, 2004). Measuring a ratio of waist to hip circumference therefore integrates the increased risk from a large waist (reflecting intra-abdominal fat) with the reduced risk of increased hip size (due to increased lean tissue).

Imaging techniques have been used to measure intra-abdominal adipose tissue more accurately. Computed tomography (CT) provides the gold standard to distinguish intra-abdominal from subcutaneous adipose tissue but the radiation exposure during a scan limits its widespread use. Dual-energy X-ray absorptiometry (DXA) and Magnetic Resonance Imaging (MRI) do not require radiation and are becoming increasingly useful to measure visceral adipose tissue (Clasey *et al.*, 1999; Bertin *et al.*, 2000; Gomi *et al.*, 2005) (Browning *et al.*, 2010). These techniques are discussed in detail in Chapter 2.

### 1.2.2 Obesity-related diseases

Concern regarding the increasing prevalence of overweight and obesity has arisen because of the association with a number of risk factors for cardiovascular disease and chronic diseases including dyslipidaemia, hypertension, type 2 diabetes, coronary artery disease, obstructive sleep apnoea, gallbladder disease, osteoarthritis, breast, endometrial and colon cancers, and fertility problems (WHO 1998) (James *et al.*, 2006). Most notably the relative risk for developing type 2 diabetes increases exponentially with an increase in BMI (Figure 1.2.1) (Colditz *et al.*, 1995) (Chan *et al.*, 1994).

**Figure 1.2.1 Body mass index and relative risk for developing type 2 diabetes modified from Colditz *et al.*, 1995 and Chan *et al.*, 1994**




Additionally, BMI is a strong predictor of mortality. In a prospective study of 900 000 adults mortality was lowest at a BMI of 22.5-25 kg/m$^2$. Each 5 kg/m$^2$ increase in BMI above this range was associated with 30% higher overall mortality, a 40% increase in mortality from stroke and vascular disease (particularly due to heart failure and hypertension), a 120% increase in diabetes mortality, 60% increase in renal disease mortality, 80% increase in liver disease mortality, 10% increase in neoplastic mortality and 20% increase in mortality from respiratory disease. At a BMI of 30-35 kg/m$^2$, median survival is reduced by 2-4 years, and at a BMI of 40-45 kg/m$^2$ it is reduced by 8-10 years, comparable to the effects of smoking (Whitlock *et al.*, 2009).

Energy regulation and the prevention of excess gain in body mass is critical to decrease morbidity and mortality related to obesity. In addition, identification of those at specific risk of disease is important in order to attempt to reduce the development of diabetes and cardiovascular disease.

#### *1.2.2.1 The metabolic syndrome*

The metabolic syndrome is a cluster of features which are associated with an increase in the risk of cardiovascular and metabolic disease (particularly type 2 diabetes) and may be the first indicator of developing weight-related morbidity. The syndrome includes abnormalities of blood lipids, blood pressure and blood glucose metabolism with obesity, particularly abdominal obesity, now being recognised as a key factor.

The prevalence of the metabolic syndrome varies with age and ethnicity and is increasing (Eckel *et al.*, 2005). Prevalence in the USA was reported as 24% in 2003 (Ford & Giles, 2003) with higher rates in older age groups (44% aged 60-69 y) and amongst minority ethnic groups. As increasing rates are being seen in all ages it is important to identify the syndrome accurately to be able to identify those most at risk of developing later disease and determine appropriate preventative strategies.

A World Health Organisation consultation proposed a set of criteria to define the metabolic syndrome in 1999 as the presence of insulin resistance together with at least two of: raised blood pressure, hypertriglyceridaemia and/or low HDL-cholesterol, obesity (as measured by waist/hip ratio or body-mass index), and microalbuminuria. In 2001, the National Cholesterol Education Program Expert Panel on Detection, Evaluation, and

Treatment of High Cholesterol in Adults (ATP III) defined the metabolic syndrome with a focus on cardiovascular risk (NHLBI, 2001). Three or more features of central obesity, hypertriglyceridaemia, low HDL cholesterol, hypertension, and elevated fasting plasma glucose defined the metabolic syndrome. A comparison of these two main definitions of the metabolic syndrome identified that, although the prevalence for the whole population is similar using either definition, 15-20% of people would be classified differently depending upon the criteria used (Ford & Giles, 2003).

A call for a unified definition of the metabolic syndrome led the International Diabetes Federation (IDF) to propose a new definition in 2004 that would reduce confusion and allow greater comparability between studies (Alberti *et al.*, 2005; Eckel *et al.*, 2005; IDF, 2005). In this new definition, central obesity is essential for determining the metabolic syndrome because of the strength of the evidence linking waist circumference with cardiovascular disease and the other metabolic syndrome components (Alberti *et al.*, 2005) and the likelihood that central obesity is an early step in the aetiology of the metabolic syndrome. Changes in features of the metabolic syndrome over 4.5 years in a prospective cohort identified obesity to be the central feature of the syndrome and suggested the importance of obesity in the aetiology of the metabolic syndrome (Maison *et al.*, 2001). While the cluster of features of the metabolic syndrome predict cardiovascular disease and type 2 diabetes (Isomaa *et al.*, 2001; Laaksonen *et al.*, 2002; Lakka *et al.*, 2002; Hu *et al.*, 2004) the importance of the link between waist circumference and cardiovascular disease (Eckel *et al.*, 2005) has led to the IDF requiring the presence of central obesity in order for diagnosis of the metabolic syndrome. The criteria for the IDF definition of the metabolic syndrome are shown in Table 1.2.1.

**Table 1.2.1 Criteria for the diagnosis of the metabolic syndrome according to the IDF definition**

| | | |
|---|---|---|
| **Central obesity**<br>Waist circumference | ≥ 94 cm for Europid men<br>≥ 80 cm for Europid women | ethnicity specific values for other groups |
| **Plus any two of:** | | |
| Raised triglycerides | ≥ 150 mg/dL (1·7 mmol/L) | or specific treatment for this lipid abnormality |
| Reduced HDL-cholesterol | < 40 mg/dL (1·03 mmol/L) men<br>< 50 mg/dL (1·29 mmol/L) women | or specific treatment for this lipid abnormality |
| Raised blood pressure | Systolic ≥ 130 mm Hg<br>or<br>Diastolic ≥ 85 mm Hg | or treatment of previously diagnosed hypertension |
| Raised fasting plasma glucose | ≥100 mg/dL (5·6 mmol/L) | or previously diagnosed type 2 diabetes |

Preventing and managing obesity therefore appear to be critical to reduce the incidence of diseases that arise from longstanding features of the metabolic syndrome. In the obese, components of the syndrome must be identified and managed.

*1.2.2.2 Glucose and insulin metabolism*

In healthy subjects, glucose levels are maintained between tight limits. After a meal insulin release from the pancreatic beta cells regulates glucose uptake from the blood to peripheral tissues. Insulin also inhibits gluconeogenesis and glycogenolysis in the liver to prevent glucose production in the post-prandial period. Dysfunction of beta cells and insulin resistance, leading to dysregulation of glucose, may occur early in the development of metabolic disease, and elevated glucose levels, particularly post-prandially, have been associated with an increase in cardiovascular risk, oxidative stress, endothelial dysfunction, a pro-inflammatory response and hypercoagulability (Ceriello, 2005; Tushuizen *et al.*, 2005).

For this reason hyperglycaemia was a key feature of the WHO diagnostic criteria for the metabolic syndrome (Alberti & Zimmet, 1998) and remains an important component. Hyperglycaemia predicts type 2 diabetes, the complications of which are a leading cause of morbidity and mortality, and is an independent risk factor for cardiovascular disease (Ceriello, 2005), (Gerstein, 1997). In the UKPDS a reduction in complications from diabetes was shown when glycated haemoglobin (a measure of advanced glycation end-products which reflect glucose levels over the preceding 6-8 weeks) was lowered, and blood glucose measured 2 hours after a 75g oral glucose load was seen to be a better predictor of all cause mortality than fasting glucose (UKPDS, 1998).

In a similar manner mild hyperglycaemia (within non-diabetes limits) has been associated with an elevated risk of cardiovascular disease and is increasingly being recognised as an important risk for developing metabolic disease. The DECODE study group identified the

importance of post-prandial hyperglycaemia in 18,048 men and 7,316 women from 13 prospective European cohort studies. Abnormalities in 2 hour glucose concentration after a 75g oral glucose load predict mortality more accurately than fasting glucose, and mortality is increased with an elevated 2 hour glucose at any level of fasting glucose including those within the normal range (DECODE, 1999). A meta-analysis of 20 studies involving more than 95 000 people identified a continuous increase in cardiovascular risk with post-prandial hyperglycamia, including normal glucose levels (Coutinho *et al.*, 1999). Identifying dietary factors that may curtail the rise in post-prandial glucose may therefore reduce the risk of cardiovascular disease and mortality.

## 1.3 Dietary determinants of body mass

The foregoing evidence illustrates the need to attempt to achieve and maintain a healthy body mass to prevent the development of obesity, and to reduce metabolic risks to limit the burden of disease in those currently obese. This thesis focuses on dietary strategies, as they are widely applicable, low in cost, relatively straightforward to implement and provide an important foundation for the maintenance of energy balance.

### 1.3.1 Characteristics of meals and eating patterns

The energy density of foods has an important role in energy regulation and increases in dietary energy density are associated with a higher energy intake (Johnson *et al.*, 2009). Energy density (kJ/g) is the amount of energy (kJ) in a given weight of food (g). Fat has the highest energy content per gram (37kJ/g) (compared with carbohydrate, 16 kJ/g and protein which provides 17 kJ/g) so a higher proportion of fat in a meal increases its

energy density and has been associated with increased total energy intake (Rolls *et al.*, 2005). Experiments with volunteers show that foods which have a higher energy density increase consumption of total energy at the meal while low energy-dense food eaten at the start of a meal, such as a salad (Rolls *et al.*, 2004), or water added to a meal (Rolls *et al.*, 1999), reduce total energy intake (Rolls *et al.*, 2005).

Portion size is another factor that may predispose to obesity. When larger portions are offered in an experimental setting, more food is consumed and individuals do not report differences in satiety with increased food intake (Ello-Martin *et al.*, 2005). Moreover, there is incomplete compensation in energy intake at subsequent meals after 2 days of increased portion size (Ledikwe *et al.*, 2005; Rolls *et al.*, 2006) or when portion sizes were increased over 11 days (Rolls *et al.*, 2007). Whether portion size has an effect on long-term energy intake remains to be determined.

Frequency of eating has also been linked to weight control but the evidence is inconsistent. In observational studies more frequent eating episodes are associated with lower body weight, however it is likely that the nature of food consumed at each episode is the most important factor in determining the effect of eating episodes on energy regulation (Drummond *et al.*, 1996).

The evidence for a relationship between dietary components and obesity is limited by the multifactorial nature of eating behaviour and food intake in humans, and confusion generated by under-reporting of energy intake which is higher in overweight and obese individuals (Livingstone & Black, 2003; Rennie *et al.*, 2005). If total energy intake is

under-reported, it is assumed that other nutrients are also underestimated so that identifying a nutrient-specific role in the development of obesity is difficult. Dietary patterns have been explored to investigate whether a broader group of food or nutrients may be more closely associated with obesity. Diets high in fat or sugar and low in fibre have been associated with obesity, while a change to a diet lower in fat and sugar and high in fibre over 10 years resulted in a reduction in BMI in obese women and less increase in BMI in lean women (Newby *et al.*, 2006). In children, high fat, low fibre, energy dense dietary patterns are associated with an increase in fat mass and linked to excess gain in body mass (Johnson *et al.*, 2008).

### 1.3.2 Macronutrient regulation of energy intake

The macronutrient composition of the diet may have a specific effect on appetite, and hence energy intake. Satiety appears to be linked to the ability to store or utilize dietary energy and so macronutrients that are tightly regulated have more effect on subsequent energy intake and energy balance (Stubbs *et al.*, 1995a; Jebb *et al.*, 1996). There is limited ability to store protein, and protein oxidation is closely linked to protein intake (Jebb *et al.*, 1996, Shetty, 1994 #568). Conversely, the ability to store fat is large, and there appears to be almost no autoregulation between fat intake and fat oxidation, leading to fat storage when intake is in excess (Prentice, 1995). Protein and carbohydrate (which also has well regulated substrate oxidation) may therefore exert more control over energy regulation than fat.

Traditional recommendations for weight control or weight loss promote high-carbohydrate, low-fat diets. These diets provide a reciprocal change in the proportion of

carbohydrate and fat, while protein remains unchanged. The focus on reducing fat intake has developed from short-term experimental studies which have demonstrated that increasing the proportion of energy from dietary fat increases hunger and total energy intake (Rolls *et al.*, 1994; Stubbs *et al.*, 1995a). Reducing dietary fat (by increasing carbohydrate) over six months has been shown to reduce weight gain and increase weight loss (Saris *et al.*, 2000; Saris, 2003; Astrup, 2006). A meta-analysis of 16 intervention studies identified a 3.2 kg greater weight loss in subjects following a low-fat diet even when energy intake was not intentionally restricted (Astrup *et al.*, 2000). The proposed mechanism for the weight loss effect is the lower energy density of the fat-reduced diet, so that this diet provides more food volume and weight for less energy, and hence greater satiating effect than a higher-fat diet. It is has also been proposed that individuals susceptible to obesity have an impaired ability to rapidly adjust fat oxidation in response to increasing the dietary fat content, therefore a diet lower in fat may be beneficial (Astrup, 2006). However, the degree of weight loss demonstrated in this meta-analysis does not persist in studies over a longer period. In the Women's Health Initiative Dietary Modification Trial, a low-fat, high-carbohydrate diet resulted in weight loss of 2.2 kg in the first year, reducing to 0.4 kg after 7.5 years (Howard *et al.*, 2006) although this was not statistically different from the control group. However, a clear dose response effect between fat reduction and weight loss evident in the post-hoc analysis, strongly suggests that the lack of an overall long-term effect is the result of poor compliance.

A recent study investigating the long term (2 year) effect of four diets: low-protein, low-fat; high-protein, low-fat; low-protein, high-fat; and high-protein, high-fat in 811 overweight adults did not demonstrate a difference between diet groups in 2 y weight loss

or change in waist circumference (Sacks *et al.*, 2009). Limited long-term compliance was undoubtedly a factor in the inability of this trial to differentiate between the four diets. Attendance at diet counselling sessions was strongly associated with weight loss, suggesting that those who followed the prescribed diet were successful at weight loss. Dietary goals were only partly achieved, differences in macronutrient intake were small and the intended reduction in energy intake was not sustained, making it difficult to draw conclusions of the specific impact of macronutrient composition.

In contrast, a similar 2 year study in 322 obese subjects investigated the effect on weight loss of three diets: low-fat, low-energy; Mediterranean, low-energy; low-carbohydrate, *ad libitum*; providing information about diets in a workplace cafeteria rather than relying on counselling sessions for dietary instruction. Weight loss was achieved in all three groups, with greater reduction in the Mediterranean diet group (4.4 kg) and the low-carbohydrate group (4.7 kg) than the low-fat group (2.9 kg) ($p<0.001$) (Shai *et al.*, 2008).

Traditionally most research has focussed on fat and carbohydrate content while keeping the percent of total energy from protein constant. Because the proportion of total energy from protein is comparatively low and protein intake over time has remained relatively constant there has been an assumption that protein was unlikely to exert significant influence over total energy intake. Newer research indicates that increasing the protein content at the expense of fat and carbohydrate might offer a way to increase the satiating and thermogenic effect of diets, and facilitate weight loss and maintenance. Experimental evidence that protein is more satiating than carbohydrate or fat (Poppitt *et al.*, 1998) has recently been accompanied by data from clinical trials examining the potential benefit of

protein in short and long-term energy intake and weight regulation (Raben *et al.*, 2003; Weigle *et al.*, 2005). Protein has higher diet-induced thermogenesis and may additionally influence weight regulation through effects on energy expenditure (Westerterp-Plantenga *et al.*, 1999). A meal high in protein may also help to reduce post-prandial glycaemic excursions and so have beneficial effects on metabolic disease risk. There is therefore a need for a fundamental understanding of the role of protein in energy metabolism, including energy intake, energy expenditure, and metabolic risk and to explore some of the underlying mechanisms. The role of dietary protein on these factors is the focus of this thesis.

## 1.4 Protein requirements

Protein requirements are based on estimates of the amount of high-quality egg or milk protein (which are completely digestible) required for nitrogen equilibrium. Additional allowances for times of increased growth (infants, children, pregnancy, lactation) are included in the recommendations and are calculated to ensure adequate weight gain or growth, a suitably positive nitrogen balance, and the maintenance of a state of well-being (COMA, 1991). The recommended daily intake for protein is presented in Table 1.4.1. In a 70 kg male this intake of 53 g protein per day is equivalent to 11% of an 8000 kJ diet. The recommendations assume that the dietary protein intake includes sufficient variety of protein-containing foods or sufficient high-quality animal protein to provide for indispensable amino acid (IAA) requirements. Previous studies in the UK have indicated that the average household consumes a diet containing adequate amounts of IAA (Buss & Ruck, 1977) and no further recommendations on the type of protein to be consumed were considered necessary. If diets contain a considerable amount of unrefined cereal grains

and vegetables a correction for digestibility of 85% should be applied.

The Committee on Medical Aspects of Food (COMA) recognises that there are a number of areas of uncertainty in the recommendations for protein intake. Maintenance needs for protein are based on achieving nitrogen equilibrium in nitrogen balance studies. These needs equate to about 70 % more than the minimum rates of nitrogen loss in individuals fed a protein-free diet. It is not understood why there is such a large discrepancy in this excess of requirements over minimum needs, however it is possible that miscellaneous losses are not adequately accounted for or calculated correction factors are incorrect. Dietary amino acids regulate growth, development and body function and may therefore be important in determining protein requirements, particularly in times of rapid growth, and may account for the excess of requirements above minimum needs.

The committee however expressed concern over high-protein intakes because of the potential for excessive protein to impair renal function. While acknowledging that there is little evidence to support this theory in healthy individuals with normal renal function, an upper limit of 1.5g protein/kg/d was suggested. In a 70 kg person this fraction equates to 105 g protein per day or 22% of an 8000 kJ diet.

**Table 1.4.1 Recommended daily allowance of protein based on recommendations from the Committee on Medical Aspects of Food (COMA)**

(COMA, 1991).

| Age | Weight<br>kg | Reference Nutrient Intake<br>g/d |
|---|---|---|
| Infants and children | | |
| 0-3 months | 5.9 | 12.5 |
| 4-6 months | 7.7 | 12.7 |
| 7-9 months | 8.8 | 13.7 |
| 10-12 months | 9.7 | 14.9 |
| 1-3 years | 12.5 | 14.5 |
| 4-6 years | 17.8 | 19.7 |
| 7-10 years | 28.3 | 28.3 |
| Males | | |
| 11-14 years | 43.0 | 42.1 |
| 15-18 years | 64.5 | 55.2 |
| 19-50 years | 74 | 55.5 |
| 50 + years | 71 | 53.3 |
| Females | | |
| 11-14 years | 43.8 | 41.2 |
| 15-18 years | 55.5 | 45.4 |
| 19-50 years | 60.0 | 45.0 |
| 50 + years | 62.0 | 46.5 |
| Pregnancy | | + 6 |
| Lactation | | |
| 0-6 months | | + 11 |
| 6+ months | | + 8 |

The focus of the recommendations is based on maintaining equilibrium or replacing losses. There is no consideration as to whether protein may have other functions in the regulation of body mass or whether there is an optimal level of protein consumption for weight maintenance or glucose metabolism.

## 1.5 Mechanisms for the effect of protein on energy regulation

### 1.5.1 Protein and satiety

Protein has the potential to act at all stages of the satiety cascade to reduce immediate and subsequent food intake. Satiety measured after high-protein preload meals has been demonstrated to be higher than after isoenergetic fat or carbohydrate meals, and in some preload studies has been associated with a reduction in energy intake. The importance of satiety over a longer period in a free-living environment was illustrated by Weigle *et al* (Weigle *et al.*, 2005). 19 subjects were placed on a weight-maintaining diet (15% protein, 35% fat, and 50% carbohydrate) for 2 weeks, followed by an isoenergetic diet (30% protein, 20% fat, and 50% carbohydrate) for 2 weeks, and an *ad libitum* diet (30% protein, 20% fat, and 50% carbohydrate) for 12 weeks. Satiety was markedly increased during the isoenergetic high-protein diet. The enhanced satiety translated into a reduction in energy intake once *ad libitum* consumption of the high protein diet commenced. Reduced energy intake was maintained until the end of the 12-week period with a mean reduction in energy of 1.8 ± 0.3 MJ/day. This implies that the benefit of increased protein in body weight is primarily achieved through appetite control mechanisms.

### 1.5.2 Protein and gastric emptying

One mechanism by which protein may regulate energy intake is to slow gastric emptying and thereby increase satiation and prolong satiety. Gastric emptying was investigated in eight subjects with type 2 diabetes who consumed a soup drink prior to a meal, with the soup or meal or neither containing additional whey protein. Gastric emptying was slowest when whey was added to the soup drink (Ma *et al.*, 2009). Park *et al* (2007) (Park *et al.*, 2007) assessed gastric emptying using scintigraphy after subjects consumed diets containing 2.1 MJ additional protein, fat, or carbohydrate, compared to a standard diet, over a two week period. No difference in the rate of solid meal emptying was observed after a standard labelled egg meal. In two studies, gastric emptying was measured after preloads of whey and casein using an oral dose of 1500 mg of paracetomol. Plasma paracetamol levels can be measured as a marker of the rate at which the paracetamol and preload meal is emptied from the stomach. Hall *et al* (2003) (Hall *et al.*, 2003), demonstrated an initial rapid gastric emptying after casein than whey, but subsequent slower gastric emptying after the casein preload. In this study, lower energy intake and greater satiety were measured after meals containing whey compared to casein which may be accounted for by the initial delay in gastric emptying resulting in increased satiety. Bowen *et al* (2006) (Bowen *et al.*, 2006c) found no difference in gastric emptying between whey and casein preloads but gastric emptying was slower after the protein preloads than after glucose and lactose preloads. A reduction in energy intake and satiety was also present after the protein preloads compared to the glucose preload consistent with the theory that delayed gastric emptying increases satiety and reduces subsequent energy intake.

### 1.5.3 Protein and gastrointestinal hormones

Hormones released from the gastrointestinal tract in response to food or metabolic signals have an important role in the regulation of food intake (see section 1.1.1.3). Stimulating anorexigenic hormones and inhibiting orexigenic hormones may be a mechanism for the increased satiety effects of protein.

#### *1.5.3.1 Ghrelin*

When liquid preloads differing in carbohydrate and protein were given to 19 overweight subjects, plasma ghrelin levels were suppressed for a longer period after a protein drink than after glucose, suggesting reduced action of the orexigenic pathways stimulated by ghrelin. Decreased energy intake at a subsequent meal was also seen after the protein drink (Bowen *et al.*, 2006b). In contrast measurements of blood ghrelin in 14 subjects showed that post-prandial release increased after fat and protein meals, and decreased after a high-carbohydrate meal, despite an associated increase in satiety (Erdmann *et al.*, 2004). Ghrelin concentrations were not suppressed during 24 hours of increased protein intake in a calorimeter (Lejeune *et al.*, 2006) or during 16 week weight loss diets (Moran *et al.*, 2005) (Weigle *et al.*, 2005) but decreased in proportion to weight loss and fasting glucose levels.

Ghrelin concentration is low when insulin levels are high immediately after a meal and it is possible that post-prandial insulin levels may mediate an apparent macronutrient effect on ghrelin release (Erdmann *et al.*, 2004). However the prolonged suppression of ghrelin after a meal occurs when insulin concentration has returned to baseline (Bowen *et al.*, 2006b) suggesting an effect independent of insulin. The pattern of release of ghrelin in

response to meals is inconsistent and the interaction with insulin, satiety and long-term energy intake requires clarification.

### *1.5.3.2 GLP-1*

Macronutrient composition appears to influence GLP-1 release and may mediate the differences in satiety seen in response to various experimental meals. In 12 lean women consuming a high-protein diet (30% of energy as protein) compared to a diet comprising 10% of energy as protein for four days, higher post-prandial concentrations of GLP-1 were observed after the high-protein diet. Additionally enhanced satiety was associated with the increase in GLP-1 levels. (Lejeune *et al.*, 2006). In contrast, Batterham *et al* found no difference in GLP-1 levels after test meals comprising 65% of energy from protein, 64% of energy from fat or 66% of energy from carbohydrate with protein comprising 17% of energy in the latter two meals (Batterham *et al.*, 2006). Protein type has also been investigated for an effect on GLP-1. When meals containing whey or casein protein were provided to human subjects GLP-1 release was 65% higher after consumption of whey, the form of protein that was found to be the most satiating (Hall *et al.*, 2003).

### *1.5.3.3 PYY*

Batterham *et al* (Batterham *et al.*, 2006) have proposed that PYY is a critical factor in protein-induced satiety and its role in regulation of body weight. In the crossover study described above, high-protein meals produced the greatest increase in PYY in human volunteers. It is interesting to note that the test meals chosen contained a proportion of protein that exceeded standard intake or conventional 'high' protein diets. To further

investigate the PYY response, mice were fed high (40%) and low (15%) protein meals. Post-prandial release of PYY was increased after the high-protein diet and enhanced PYY synthesis and secretion was observed after 16 weeks of *ad libitum* feeding on the high protein diet. In a second group of mice the PYY coding region was deleted. These mice developed marked obesity which was reversed by exogenous PYY, suggesting the importance of PYY in energy homeostasis.

#### *1.5.3.4 CCK*

CCK levels have been shown to increase in response to a protein load. Hall *et al*, 2003 identified an increase in CCK after whey protein meals compared with casein associated with enhanced gastric emptying (Hall *et al.*, 2003). Higher CCK secretion was similarly seen after whey in comparison to casein, glucose and lactose and was correlated with satiety but not energy intake (Bowen *et al.*, 2006b). CCK may therefore mediate the satiating properties of dietary protein but as is seen in other preload studies enhanced satiety may not translate into effects on long-term energy regulation.

#### *1.5.3.5 PP*

Stimulation of PP has been observed in response to protein meals in adults (Tomita *et al.*, 1989) due in part to increased circulating levels of amino acids (Schmid *et al.*, 1989). 19 obese children provided with a low (0.2 g/kg) and high (2.0 g/kg) protein meal demonstrated enhanced post-prandial secretion of PP after the high-protein meal (581 pg/ml) than the low-protein meal (302 pg/ml) (Zipf *et al.*, 1983). Neural and other endocrine factors appear to contribute to the release of PP (Karhunen *et al.*, 2008) as is evidenced by a higher concentration of blood PP after sham fed meals high in protein

compared to meals high in carbohydrate (Witteman *et al.*, 1994).

### 1.5.4 Protein and energy expenditure

Protein may also modulate energy regulation by increasing energy expenditure, so favouring weight loss. The success of the Atkins diet (the popular high-protein, low-carbohydrate, high-fat diet) has been attributed to weight loss occurring because of a 'metabolic advantage' from the combination of reduced digestible energy, increased diet-induced thermogenesis, enhanced macronutrient oxidation, and loss of energy in the form of ketones (Atkins, 1998). There is little direct evidence for these effects; however, enhanced weight loss in clinical trials of high-protein diets (Samaha *et al.*, 2003) or maintenance of weight loss (Westerterp-Plantenga *et al.*, 2004) is reported to occur without a measured difference in reported energy intake. This failure to account for weight loss through reduced energy intake may be in part due to misreporting of food intake, but may also support the theory of enhanced energy expenditure with an increased protein to energy ratio.

Protein may influence energy expenditure by increasing one or all of the components of 24-hour energy expenditure – basal metabolic rate, diet-induced thermogenesis, and activity-induced energy expenditure. Differences in absorption of protein compared with other macronutrients, and effects on substrate oxidation may also be factors that affect energy balance and therefore weight regulation.

Diet-induced thermogenesis (DIT) was 17% higher after a high-protein meal (32% of energy from protein) than after meals rich in fat or carbohydrate (12% of energy from

protein) (Raben *et al.*, 2003) and repeated elevations in DIT have been observed when a high-protein diet is consumed throughout a single day (Westerterp *et al.*, 1999). However these effects cannot be clearly linked to an increase in total energy expenditure or sleeping metabolic rate over 24 hours (Westerterp *et al.*, 1999) probably because in humans DIT is quantitatively a small proportion of total energy expenditure. After 4 days following a high-protein diet, healthy overweight subjects showed a significant increase in DIT that was associated with a 3% increase in 24 h energy expenditure, suggesting adaptation had occurred that further increased energy expenditure (Mikkelsen *et al.*, 2000; Lejeune *et al.*, 2006). Effects on energy expenditure differed with protein type, with increased energy expenditure on diets high in animal versus vegetable protein (Mikkelsen *et al.*, 2000).

Changes in energy expenditure may contribute to changes in body mass in people eating high-protein diets but are unlikely to account for the difference entirely. Weight lost over six months in intervention studies was approximately 4 kg greater in subjects eating the high-protein diet than in those eating a diet with adequate protein (Foster *et al.*, 2003; Samaha *et al.*, 2003). The absolute difference in total energy expenditure in the above studies range from 0.24 MJ/d to 0.49 MJ/d which would produce a 43.7 MJ to 89.2 MJ energy deficit over six months. Assuming body mass lost comprises 75-80% as fat and 20-25% as lean tissue, a deficit of approximately 29 MJ is required to lose 1 kg. Therefore 1.35 kg to 2.75 kg over six months could be accounted for by energy expenditure based on the difference in 24-hour energy expenditure seen in these studies. In longer, community-based interventions, increasing dietary protein has not produced a significant effect on total energy expenditure in subjects with hyperinsulinaemia

(Luscombe *et al.*, 2003), or on BMR and DIT in subject with type 2 diabetes (Luscombe *et al.*, 2002), or on BMR during weight maintenance after weight loss (Westerterp-Plantenga *et al.*, 2004).

### *1.5.4.1 Nutrient absorption*

All macronutrients except fibre-rich carbohydrates are well absorbed after ingestion, although protein has a lower proportion of digestible energy (91%) compared to fat (96%) (Southgate & Durnin, 1970). This difference in absorption is a small percentage of total energy consumed so does not produce a clinically significant effect on energy balance (Prentice, 1995). For example if protein provides 15% of energy from an 8 MJ/day intake, total energy comprises 1.2 MJ of protein of which 1.09 MJ (91%) is absorbed. Increasing the proportion of energy from protein to 30% results in 2.4 MJ intake from protein. With this increase in the proportion of energy from protein, an extra 0.11 MJ/day would not be absorbed, leading to a 19 MJ deficit over six months. Using the above calculation, this decrease in energy intake would result in 0.65kg weight loss in six months that could be attributed to lower nutrient absorption. This quantity is insufficient to account for more than a fraction of the observed weight loss in clinical trials.

### *1.5.4.2 Substrate oxidation*

Substrate oxidation occurs in a clear hierarchy inversely related to the ability of the body to store macronutrients (Jebb *et al.*, 1996). Carbohydrate is stored as glycogen and because there is limited capacity for glycogen storage oxidation is tightly regulated to intake. Similarly, protein has few sites for storage and protein oxidation is also well

regulated (Shetty *et al.*, 1994). In contrast, fat is easily stored in adipose tissue and little regulation occurs when fat intake alters. Metabolic fuel selection was investigated in 6 lean men who overate (16.5 MJ/d) or underrate (3.5 MJ/d) for 12 days while confined in a whole-body calorimeter. Carbohydrate intake dominated substrate oxidation with tight autoregulation such that carbohydrate balance was re-established after 2-4 d of energy imbalance, despite large differences in intake. Protein was less well regulated. Oxidation of protein increased during overfeeding and decreased during underfeeding but protein balance was not achieved during the 12 days of investigations. Conversely, fat oxidation occurred in a reciprocal manner to intake, dependent on the need to maintain carbohydrate balance. During overfeeding, fat oxidation fell rapidly from baseline in the first few days, suppressed by the increase in carbohydrate oxidation. In contrast, fat was required to be the major oxidative fuel during underfeeding, and fat oxidation was substantially higher than intake throughout (Jebb *et al.*, 1996). The counterregulation of fat oxidation, due to the dominance of carbohydrate in the oxidative hierarchy, leads to fat storage and weight gain when fat is consumed in excess (Prentice, 1995). Studies to date have investigated diets with a standard intake of protein (15% of energy) and the effect of altering the proportion of protein on metabolic fuel selection is not known.

## 1.6 Protein and energy regulation in human studies

The foregoing evidence suggests a number of plausible mechanisms by which protein affects energy regulation and body mass. The predominant mechanism is likely to be due to an effect on appetite and energy intake, and studies investigating this relationship are discussed below. Energy expenditure appears to increase after a protein rich meal, however there remains uncertainty about whether this effect persists in the long-term or is

of sufficient magnitude to make a meaningful difference to energy balance.

### 1.6.1 Studies of high-protein meals in a preload design

A number of studies have investigated the satiating properties of protein using a preload design as summarised in Table 1.6.1. There is a suggestion from these data that when comparing macronutrient preloads, high-protein (P) meals reduce hunger and subsequent energy intake to a greater degree than isoenergetic high-fat (F) or high-carbohydrate (C) meals (Barkeling *et al.*, 1990; Porrini *et al.*, 1995; Poppitt *et al.*, 1998; Vozzo *et al.*, 2003). For example, a 12% reduction in energy intake was observed 90 min after a 2 MJ high-protein preload lunch (60% P, 20% C, 20% F) compared to a high-carbohydrate meal (11% P, 68% C, 19% F) (Poppitt *et al.*, 1998), and 4 h after a 2.6 MJ lunch energy intake was similarly reduced by 12% with a high-protein meal (43% P, 37% C, 22% F) compared to a high-carbohydrate lunch (10% P, 69% C, 22% F) (Barkeling *et al.*, 1990). A 12 % difference in energy intake over 7 h of *ad libitum* eating was observed after a high-protein preload yoghurt drink (29% P, 44% C, 24% F) compared to a high-carbohydrate drink (14% P, 60% C, 24% F) (Vozzo *et al.*, 2003). Larger effects on energy intake have been seen after whole-food meals or liquid preloads when protein provided a high-proportion of energy (24% reduction in energy intake with a 71.5% protein drink (Latner & Schwartz, 1999), 46% lower energy intake after a 56% protein meal (Porrini *et al.*, 1995)). Oesch *et al.* (2005) found total energy intake (of a preload and subsequent test meal) was reduced by 19% with a protein preload (containing 98% protein, 912 MJ), compared to a water control, and was reduced by 27% with the addition of an intraduodenal infusion of fat (Oesch *et al.*, 2005). The protein preload significantly

reduced the eating time at the test meal which may have accounted for the reduction in energy intake.

Other studies have failed to show an effect on energy intake with differences in preload macronutrient composition. Stubbs *et al.* (1996), provided meals high in protein (59% of energy as protein), carbohydrate (61% of energy as carbohydrate) and fat (57% of energy as fat) to 6 men resident in a calorimeter in a crossover study (Stubbs *et al.*, 1996). Meals were whole foods presented in a similar way to a standard meal and were of similar energy density. No difference in energy intake was seen at the following meal or over the whole day but hunger scores were lower after the high-protein meal was consumed. However the small number of subjects suggests this study may have been statistically underpowered. In a similar design Raben *et al.* (2003) provided 20 lean male and female subjects with whole food meals rich in protein, carbohydrate, fat or alcohol with the same energy density and fibre (Raben *et al.*, 2003). In the high-protein meal, protein provided 31.8% of energy, more than twice the recommended proportion of protein but lower than that used in other preload studies. The protocol illustrates the difficulty in obtaining a higher percentage of energy from protein without using modified food. Neither energy intake at a subsequent meal or satiety following the meal differed between the meals. However, it is notable in these studies that the meals were of the same energy density (Stubbs *et al.*, 1995b, Raben, 2003 #23) and fibre (Raben *et al.*, 2003), perhaps suggesting that the impact of protein on satiety is not independent of other dietary factors.

Preload studies therefore suggest that an increase in the proportion of protein enhances satiety and, have in some experimental settings, led to a subsequent reduction in energy

intake at the next meal or over course of day. A possible explanation for the discrepancy between macronutrient effects on satiety and subsequent energy intake is the complex interaction between behaviour and physiological control of appetite. While innate physiological signals may indicate the onset of satiation, they may need to be strong to overcome other learnt behaviours such as energy consumed during a meal (Poppitt *et al.*, 1998).

**Table 1.6.1 Single Meal Preload Studies**

| | | STUDY DESIGN | | RESULTS | | |
|---|---|---|---|---|---|---|
| **Reference** | **Subjects** | **Intervention** | **Preload meal** | **Appetite measures** | **% Change in Energy Intake** | **Other Results** |
| Stubbs *et al*, 1996 | n = 6<br>Lean males<br>crossover | Day 1 maintenance diet EI 1.5xRMR<br>13% P, 47% C, 40% F<br>Day 2 in calorimeter<br>Preload breakfast 0830, EI 75% of RMR<br>HP 59% P, 22% C, 19% F<br>HC 18% P, 61% C, 21% F<br>HF 21% P, 22% C, 57% F<br>Similar energy density<br>Test meal at 5 h (1330) | Steak, potatoes, sweetcorn, mushrooms, gravy, fruit, yoghurt | HP and HC less hunger than HF<br>mean whole day hunger score lower for HP less than HC/HF | No diff in EI at test meal or over whole day | EE no sig diff<br>Oxidation – P increased with HP meal, C increased with HC meal<br>fat oxidation highest with HF > HP > HC |
| Raben *et al*, 2003, | n = 20<br>lean female and male | Day 1 standard diet 13.2% P, 49.2% C, 37.4% F<br>Day 2<br>Preload breakfast 2500 kJ F, 3000 kJ M<br>HP 31.8% P, 37.2% C, 31.1% F<br>HC 12.2% P, 65.4% C, 23.7% F<br>HF 11.6% P, 23.9% C, 64.6% F<br>Alcohol<br>12.1% P, 42.9% C, 24.3% F, 23% Alcohol<br>Energy density and fibre constant | HP Cheese, crispbread, muesli, yoghurt, boiled egg, skim milk<br>HC corn flakes, skim milk, white bread, butter, cheese, jam, honey HF | Satiety/hunger – no diff<br>Higher desire for something sweet after HP<br>Higher EE with HP over whole day (remained high at end of test day)<br>Diet-induced thermogenesis 17% higher after HP vs. HC (NS) | EI no difference | Post -prandial blood samples<br>Glucose: C>P=F<br>Lactate: C>P=F<br>Insulin AUC C>P>F<br>Glucagon AUC P>F>C<br>Triacylglycerol F>P=C<br>NEFA (level below fasting) P<C<F<br>Leptin (level below fasting) F<C=P |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | BF 0945 Test meal 5 h (1500)<br>Blood samples in 10 subjects<br>Fasting samples at 30 min intervals<br>5 hours spent under ventilated hood<br>urine nitrogen measured | yoghurt, double cream, grated apple, honeydew melon, rye bread, butter, cream cheese, whole milk<br>Alcohol: rye bread whole-grain bread, butter, cheese, yoghurt, muesli, honeydew melon, orange juice with vodka | (NS) | | F<C=P<br>GIP AUC F>C>P<br>GLP-1 AUC P>C>F<br>GLP-2 AUC P=F>C |
| Poppitt *et al*, 1998 | n = 12<br>lean females<br>crossover | Standard breakfast at 0900<br>Preload Meal at 1200 approx. 2 MJ<br>HP 60% P / 20% C / 20% F<br>HC 11% P / 68% C / 19% F<br>HF 11% P / 21% C / 68% F<br>Alcohol 11% P / 21% C / 20% F /47% alcohol<br>Test meal at 90 min (1330) | Fish and potato pie and preload drink | HP less hunger, more satiety<br>HP meal least pleasant, HF most pleasant | EI after protein meal lower than after<br>Alcohol - 20.8% lower<br>HC - 12.3% lower<br>HF - 14.2% lower | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Energy density differed | | | | |
| Barkling *et al*, 1990 | n = 20<br>lean females<br>crossover | Preload lunch 612 kcal, similar fibre<br>HP 43% P, 37% C, 22% F<br>HC 10% P, 69% C, 22% F<br>*ad lib* evening meal - amount eaten measured by computer eating monitor (VIKTOR) | Meat and spaghetti vs. veg and spaghetti<br>*Ad libitum* meal | No difference in hunger, fullness, desire to eat.<br>HP more pleasant, ate slower initially | HP ate 12% less than HC | |
| Vozzo *et al*, 2003 | n = 16<br>lean males<br>crossover x4 | 3000 kJ preload meal then ad lib eating for 7 h<br>HP 29% P, 44% C, 24% F<br>HC 14% P, 60% C, 24% F<br>HF 14% P, 42% C, 40% F<br>no preload | Yoghurt based<br>500g<br>HP 29%<br>HC 60%<br>HF 40% | No difference in time to first eating or amount at first eating episode, or in hunger between macronutrients. | EI reduced compared to 'No preload'<br>HP 29%, HF 20%, HC 17% lower than No preload.<br>No significant difference between preloads but EI 12% less after HP than HC (NS) | Most consumed only one meal in 7 h<br>Excluded if<br>- >15 Eating Attitudes Test for eating disorders,<br>- >10 Three factor eating questionnaire to ensure unrestrained eaters,<br>- >50 Zung depression scale<br>Appetite assessed by VAS |
| Latner and Schwartz, 1999 | n = 12<br>females BMI 19-29 kg/m$^2$<br>crossover x3 | 450kcal lunches, ad lib dinner 4.5 h later<br>HP 71.5% P, 9.5% C, 9.6% F<br>HC 0% P, 99% C, 0% F<br>mixed 35.7% P, 55.1% C, 9.6% F<br>controlled flavour, volume, visual appearance | liquid flavourless drinks | Hunger less after HP than HC or mixed | EI after HP 24% lower than after HC.<br>EI after mixed meal 17% lower than HC | |
| Porrini *et al*, 1995 | n = 12<br>lean males<br>crossover | Preload HP / HC<br>Meals designed to differ in P and C and sensory properties<br>HP 54% P, 19% C, 25% F | Pasta vs. meatballs | Fullness, satiety higher after meatballs | EI after HP 46% lower | Satiety Q validated |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | HC 17% P, 55% C, 27% F<br>3 sessions per meal<br>1. pasta 960 kcal, meatballs 880 kcal<br>2. pasta 480 kcal, meatballs 440 kcal<br>3. session 1 then ad lib meal | | | | |
| Porrini *et al*, 1997 | n = 14 (a)<br>n = 10 (b)<br>n = 14 (c)<br>lean meals<br>crossover | Standard breakfast 0830 h<br>Omelette at 4 h (1245) as a<br>a) uniform meal (no subsequent meal)<br>b) preload immediately before a buffet meal<br>c) snack 2 hours before a buffet meal | HP a) 5.24MJ offered<br>b) 1.70 MJ large<br>0.85 MJ small<br>c) 1.15 MJ<br>P 53.5%<br>C 1.3%<br>F 45.2%<br>HF a) 5.99 MJ offered<br>b) 3.02 MJ large<br>1.51 MJ small<br>c) 1.19 MJ<br>P 14.8%<br>C 5.9%<br>F 79.3% | VAS higher fullness and satiety and lower desire to eat after the HP preload snack than the HF | EI<br>a) 42% less of the HP omelette consumed (1.72 MJ) than HF omelette (2.99 MJ)<br>b) 22% lower after large HP than after HF,<br>16% lower after small HP than after HF<br>c) No difference in EI at test meal after the snack | Weight of food consumed similar in all experiments in all conditions |
| Oesch *et al*, 2005. | n = 20<br>lean males<br>crossover | Continuous intraduodenal perfusion<br>of fat or saline given throughout experiment.<br>Oral preload of either 400 ml of water or 400 ml of protein at 40 minutes. | ID infusion 41 g of fat;<br>total energy content: 371 kcal | VAS for hunger and fullness at 15-min intervals<br>Increased fullness and reduced hunger after protein, | EI><br>19% lower after saline/protein than saline/water | 20-min intervals for plasma<br>CCK, GLP-1, and PYY<br>CCK, PYY, or GLP-1 not stimulated by protein preload |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Test meal at 60 minutes. | Protein preload:<br>0.9 MJ<br>P 98%<br>C 0.5%<br>F 2% | not affected by fat. | 27% lower after fat/protein than saline/water with reduced eating time at test meal | CCK increased after fat |

Abbreviations: P, Protein; C carbohydrate; F, Fat; HP, High Protein; HC, High Carbohydrate; HF, High Fat; EE, Energy Expenditure; EI, Energy Intake; AUC, Area Under the Curve; NEFA, Non-Esterified Fatty Acids; AA, Amino acids; CCK, Cholecystokinin; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, Glucagon-like peptide 1; GLP-2, Glucagon-like peptide 2; VAS, Visual analogue scale; NS, Non significant; RMR Resting metabolic rate.

### 1.6.2 Protein-rich diets and long term energy regulation

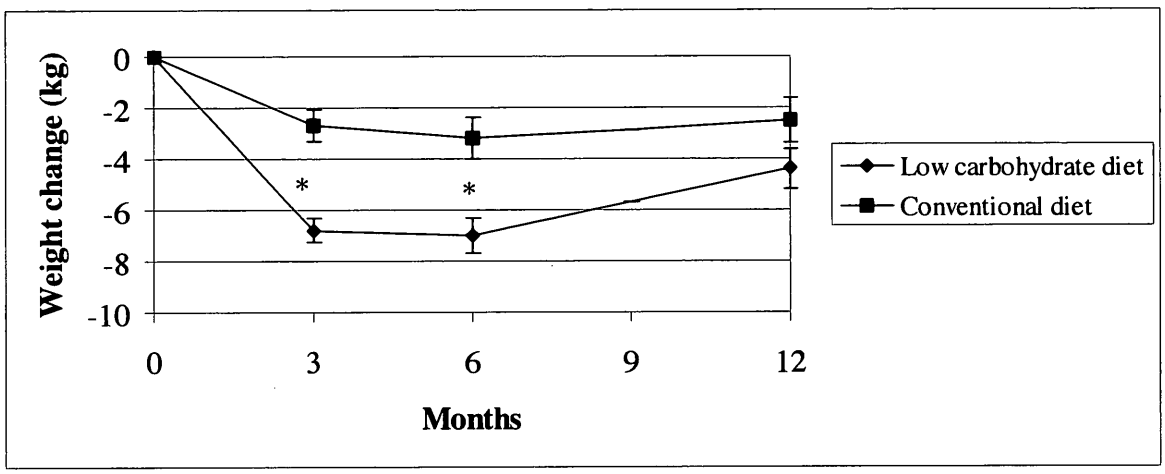
Popular weight loss programmes have in part fuelled the debate regarding the optimal level of protein consumption and whether protein intake is important in long-term weight regulation. Increasing the proportion of protein is a component of many popular diet plans. Perhaps the most prominent of these has been the Atkins diet (Atkins, 1998), where the main focus is restricting carbohydrate with fat and protein intake being significantly higher than average intakes. Anecdotal reports of enhanced weight loss have been attributed to a variety of proposed mechanisms, including effects on energy intake and expenditure. The widespread adoption of these diets among people with obesity and metabolic disease led to randomised controlled intervention studies aiming to determine their efficacy and safety.

When the Atkins diet was adopted by community-based free-living obese subjects with either minimal dietary advice (n = 63) (Foster *et al.*, 2003) or regular dietary instruction (n = 132) (Samaha *et al.*, 2003), weight loss was greater in the Atkins group after 6 months (-7.0% ± 0.8 vs. –3.2% ± 0.7 (minimal advice) and –4.5% ± 0.8 vs. 1.44% ± 0.2 (regular advice) but was not sustained at 12 months (Figure 1.6.1) (Stern *et al.*, 2004). The early weight loss was not explained by a reported difference in energy intake suggesting either differential mis-reporting of energy intake between groups or an intrinsic effect of the diet on energy expenditure. As with other low-carbohydrate diets, triglycerides were reduced but there was no other significant difference in risk factors for the metabolic syndrome or cardiovascular disease.

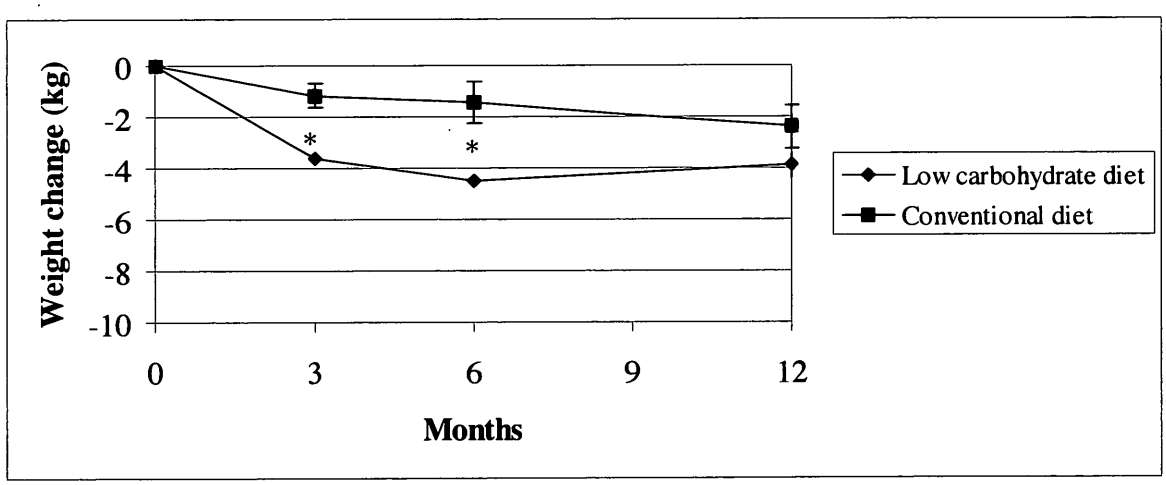
**Figure 1.6.1 Community-based free-living studies of low-carbohydrate diets showing weight loss over 12 months**

a) with minimal intervention (Foster *et al*, 2003) and b) with regular dietary instruction in severely obese subjects (Samaha *et al*, 2003, Stern *et al*, 2004). Asterisks indicate a significant difference ($p<0.05$) between the groups.

**a)**




**b)**



A recent systematic review demonstrated that low-carbohydrate Atkins-type diets (and hence high protein diets) were more effective at reducing weight and improving cardiovascular disease risk (assessed by favourable effects on high-density lipoprotein cholesterol, triacylglycerols and systolic blood pressure) than low fat, low energy diets over 6 months. Additionally, low-carbohydrate, high-protein diets were at least as effective than low-fat diets up to 1 year (Hession *et al.*, 2009).

A number of controlled dietary intervention studies have explored the effect of increasing the proportion of protein in the diet with modest reductions in fat and carbohydrate rather than the very large reduction in carbohydrate seen in Atkins-type diets. In a group of 65 overweight and obese subjects who were randomly assigned to a high-protein (25% of energy as protein), high-carbohydrate (12% of energy as protein) or control group for six months, with all food provided by a study supermarket, weight loss was greater in the high-protein group (8.7 kg CI 7.3-11.9 kg) than the-high carbohydrate group (5.0 kg CI 3.6-6.4 kg). Blood triacylglycerols and fatty acids were also reduced in the high-protein group (Skov *et al.*, 1999). A similar design was extended to twelve months in 50 overweight and obese subjects. After 12 months, weight loss was not significantly different (high protein 6.2 kg CI 3.8-8.6, high carbohydrate 4.3 kg CI 2.2-6.4), but the high protein group had a 10% greater reduction in intra-abdominal adipose tissue and 17% had lost more than 10 kg. None of the control group achieved this degree of weight loss (Due *et al.*, 2004). In a second study 100 women were assigned a 5.6 MJ/day high-protein (34% of energy from protein) or high-carbohydrate diet (17% of energy from protein) for twelve weeks. Weight loss was not significantly higher in the high-protein group (7.6 ± 0.4 versus 6.9 ± 0.5 kg). The high-protein diet had a beneficial effect on

reducing triacylglycerol concentrations and weight loss was associated with similar improvements in fasting LDL-cholesterol, HDL-cholesterol, glucose, insulin, free fatty acid, and C-reactive protein concentrations in both groups (Noakes *et al.*, 2005). A further 12 week ad libitum high-protein diet of 30% protein, 20% fat, and 50% carbohydrate produced a significant reduction in energy intake with a 4.9 kg weight loss and lower adipose tissue mass (3.7 kg) (Weigle *et al.*, 2005).

The long-term weight maintenance effect of a high-protein diet was investigated in 130 obese subjects who were assigned a high-protein (30% of energy from protein) or conventional (15% of energy from protein) diet for a 4-month period of weight loss followed by 8 months of weight maintenance. After 4 months the high-protein group had lost 22% more adipose mass than the conventional group although weight loss did not differ between the groups. At 12 months, in the group of subjects who had achieved greater than 10% weight loss (and were therefore felt to have had better compliance to the study diet), the change in weight was greater in the high-protein group (-16.5 kg) than in the conventional group (-12.3 kg) and a greater reduction in adipose mass was observed. Additionally there were fewer dropouts in the high protein diet group and greater reduction in triacyglycerols than the conventional diet (Layman *et al.*, 2009).

In the Diet Obesity and Genes study (DiOGenes), the largest study to date, 773 adults who had lost at least 8% of their body weight during a low calorie diet were randomised to one of 5 *ad libitum* dietary interventions to prevent weight regain over 6 months. The diets were low protein with low or high glycaemic index or high protein with low or high glycaemic index, or a control diet. The diets high in protein or low in glycaemic index

had lower drop outs than the low protein high glycaemic index group, and weight regain was 0.93 kg lower in high protein than low protein diets (Larsen *et al.*, in press).

These studies suggest a beneficial effect of high protein diets on weight loss, and possibly on weight maintenance, although to date the maximal effect on body mass occurs at around 6 months possibly due to compliance to the study diet. Additionally there appears to be beneficial effects on a number of metabolic risk factors, most notably reductions in fat mass and triacylglycerol levels.

## 1.7 Protein and metabolic risk

### 1.7.1 Effect on nutrient partitioning

Changing the ratio of protein to energy intake has the potential to alter nutrient partitioning and therefore body composition. Animal models have shown that increasing dietary protein delays weight gain and reduces the proportion of adipose gained (Klaus, 2005). An improvement in body composition with a reduction in the proportion of adipose mass has been shown in human studies with additional dietary protein in energy restricted diets (Layman *et al.*, 2005), (Layman *et al.*, 2003), during *ad libitum* eating (Westerterp-Plantenga *et al.*, 2004), (Weigle *et al.*, 2005) and is seen in habitual consumers of high-protein diets (Merchant *et al.*, 2005) (see also section 4.1.2.1).

Changes in body composition associated with an increase in dietary protein may be due to a reduction in adipose mass or sparing of muscle protein, or both. A reduction in post-prandial insulin after a high-protein meal may promote lipolysis and hence reduce

adipose mass. Additionally increased dietary protein reduces nitrogen losses associated with energy restricted diets and muscle protein synthesis is maintained (Layman *et al.*, 2003). Adiposity, particularly intra-abdominal adiposity, is associated with an increased risk of cardiovascular disease and is essential to the diagnosis of the metabolic syndrome (Eckel *et al.*, 2005). Altering the ratio of lean-to-adipose tissue during weight loss or weight maintenance may therefore have long-term health benefits.

### 1.7.2 Glucose and insulin metabolism

The effect of protein on blood levels of glucose and insulin has been investigated to explore whether different post-prandial responses may be associated with differences in subsequent energy intake. Fluctuating levels of glucose and insulin stimulate hunger, particularly when there is a period of hypoglycaemia following a high-glucose peak (Bray *et al.*, 1998). An increase in the post-prandial insulin AUC reflects the secretion of insulin in response to high post-prandial glucose and has been associated with reduced satiety (Holt & Miller, 1995). Stabilising post-prandial glucose and insulin levels could therefore lead to a reduction in post-meal hunger and subsequent energy intake. Post-prandial hyperglycaemia is also an important component of the metabolic syndrome and, as discussed previously, is associated with an increase in the development of type 2 diabetes, cardiovascular disease and mortality.

Raben *et al*, 2003 (Raben *et al.*, 2003) demonstrated that glucose and insulin curves are significantly lower after a high-protein meal than after a carbohydrate-rich meal, and are similar to high-fat meal. The effect of high-protein diets on glucose metabolism were investigated over a longer period using a five-week crossover design in which eight men

with untreated type 2 diabetes were provided with a diet high in protein (30% P, 20% C, 50% F) or a control diet (15% P, 55% C, 30% F). Mean 24 h serum glucose at the end of the high-protein diet was 7 mmol/l compared to 11mmol/l after the control diet. Glycated haemoglobin was also reduced after the high-protein diet (Gannon & Nuttall, 2004).

Although post-prandial or mean 24 h glucose is reduced after a high-protein diet compared to diets high in carbohydrate, it is difficult to ascertain whether the glucose lowering effect is due to an intrinsic property of protein or secondary to the reduction in carbohydrate. As current dietary guidelines for people with or without diabetes recommend a diet high in carbohydrate, recognising that replacing protein for carbohydrate produces beneficial effects on glucose metabolism is perhaps more clinically important then the underlying mechanism.

*1.7.2.1 Regulators of glucose homeostasis – IGF-I, cortisol,*

Other metabolic factors influenced by a high-protein weight maintenance diet may regulate post-prandial glycaemia, in particular elevations in growth hormone and Insulin like growth factor-1 (IGF-I), and urinary cortisol associated with elevated adrenocorticotropic hormone (ACTH) (indicating that the increased cortisol release is determined by pituitary or hypothalamic action) (Nuttall *et al.*, 2003). IGF-I has emerged as an important determinant of glucose homeostasis (Sandhu *et al.*, 2002) and may therefore potentially have a role in stabilising post-prandial glucose excursions with subsequent effects on appetite. More than 90% of IGFs in the circulation are bound to IGF-binding proteins (IGF BPs) mainly IGF BP-3. Circulating IGF-I levels are largely determined by genetic factors and age but are also affected by gender, physical activity,

diet and nutritional status (Yu & Rohan, 2000). Changes in glucose metabolism, which occur during diets differing in protein content, may therefore be partially mediated by alterations in IGF-I or its predominant binding protein, IGF-BP3.

### 1.7.3 Blood lipids

High-protein diets are associated with a reduction in blood triacylglycerols and an increase in HDL cholesterol, particularly in the setting of very low-carbohydrate, high-fat, and high-protein (Nordmann *et al.*, 2006), (Foster *et al.*, 2003), (Samaha *et al.*, 2003), (Stern *et al.*, 2004) (Noakes *et al.*, 2005; Volek *et al.*, 2005). As individuals in these studies often lost more weight following the low-carbohydrate diet, it was unclear whether reduced body mass was the main cause of any changes in blood lipid levels. In 118 subjects, randomised to an energy restricted low-carbohydrate diet (4%, 35%, and 61% of energy as carbohydrate, protein, and fat, respectively) or a low-fat diet 46%, 24%, and 30% of energy as carbohydrate, protein, and fat, respectively), designed to ensure similar weight loss in the two groups over 1 year, subjects in the low-carbohydrate group had a greater decrease in blood triacylglycerol and an increase in HDL cholesterol. Concerningly an increase in LDL cholesterol was apparent. As high LDL may be associated with an increase in cardiovascular disease risk, questions remain regarding the safety of a high-protein, low-carbohydrate, low-fat diet. (Brinkworth *et al.*, 2009).

In a comparison between a high-protein diet (30% of energy from protein) and a control diet (15% of energy from protein) when both were low in fat (30% of energy from fat), 130 subjects were randomised to one of the diets for a 4 month period of energy restriction followed by 8 months of weight maintenance (Layman *et al.*, 2009). Subjects

consuming the high-protein diet had favourable reductions in serum triacylglycerol and elevations in HDL cholesterol at 4 months and at 12 months. LDL cholesterol was elevated at 4 months in the high-protein group but no difference remained between the groups at 12 months, suggesting that in the presence of low-fat intake a high-protein diet has beneficial effects on blood cholesterol which are not attenuated by the deleterious increase in LDL seen in high-fat, high-protein diets. Similar reductions in blood triacyglycerols have been reported in previous studies of high-protein, low-fat diets (Skov *et al.*, 1999), (Layman *et al.*, 2003).

Triacylglycerols are associated with cardiovascular events (Bansal *et al.*, 2007) (Nordestgaard *et al.*, 2007) and the promotion of atherosclerosis, therefore the reduction in levels observed in high-protein diets may have positive long-term effects on cardiovascular disease. Little is currently known about the effect of increases in the proportion of protein on triacylglycerols.

### 1.7.4 Blood pressure

Blood pressure falls with weight loss; therefore investigating the effect of a particular diet when weight change is the primary outcome (as in the majority of trials comparing high and low-protein diets) is difficult. A meta-analysis of very low-carbohydrate, high-protein, high-fat diets compared with low-fat diets concluded that there was no difference between the diets in systolic or diastolic blood pressure after 1 year (Nordmann *et al.*, 2006). Similarly when high-protein, low-fat diets are compared to low-protein diets blood pressure appears to fall as a result of weight loss independent of the diet composition (Brinkworth *et al.*, 2009) However, in 60 subjects who followed either an isoenergetic

high protein (24% of energy from protein) or habitual protein (19% of energy from protein) diet for 8 weeks systolic blood pressure was 5 mmHg lower in the high-protein group (Hodgson *et al.*, 2006). As high blood pressure is an important risk factor for cardiovascular disease this finding may have important implications for the effect of protein on metabolic risk.

## 1.8 Summary

Regulation of body mass is a tightly controlled balance between energy intake and energy expenditure. Complex physiological processes initiated after the consumption of a meal control the sensations of satiation and satiety to modulate immediate food intake and future eating episodes. Energy expenditure may also be altered to maintain energy balance. Poor energy regulation is becoming an increasing problem in the developed and now the developing world, the predominant concern being a worldwide epidemic of obesity and it seems plausible that the strong biological factors that exist to regulate body mass may be over-ridden by social, cultural and psychological factors, resulting in energy imbalance. Obesity is associated with a wide range of diseases, particularly an increased risk of the metabolic syndrome, and a predisposition to type 2 diabetes, cardiovascular disease and premature death.

Dietary factors have been intensively studied to identify the specific risk factors for obesity and to establish dietary guidelines for maintenance of body mass and management of obesity. Dietary strategies to optimise energy balance must not only promote weight loss or weight maintenance, but must attempt to reduce obesity-related

disease. While the type and proportion of fat and carbohydrate have been extensively investigated for their role in weight management and disease modification, protein has only recently been identified as a potential important regulator of body mass and metabolic risk. Current evidence suggests that diets with a high protein to energy ratio increase weight loss in the obese, at least in the short-term. There needs to be a clearer understanding of the possible mechanisms involved in order to identify whether promotion of a diet high in protein is an appropriate method to achieve sustained weight loss and thereby reduce the complications associated with overweight and metabolic disease.

## 1.9 Aims and objectives

The aim of this thesis is to perform a detailed investigation into the relationship between dietary protein and body mass regulation. The specific objectives are:

- To consider the effect of protein amount and type on short-term appetite control using a classic preload design (Chapter 3).
- To investigate whether there is a dose-dependent relationship between protein consumption and energy intake in a medium-term controlled intervention study (Chapter 4), and explore in detail the role of protein in appetite control mechanisms, metabolic risk factors, and body composition.
- To determine the effect of protein on energy expenditure in a short-term highly controlled experiment (Chapter 5).

Collectively these data contribute to the evidence base to develop dietary recommendations for protein with respect to the prevention and management of obesity and associated metabolic diseases.

# 2 Chapter 2 Methods

This chapter reports on the methods available for measuring appetite, energy balance, body composition and markers of metabolic risk and describes the selection of methods used in the studies for this thesis.

## 2.1 Facilities

The study reported in Chapter 3 in this thesis was performed at the Medical Research Council Human Nutrition Research Unit (HNR) and the studies reported in Chapters 4 and 5 were conducted at HNR and the Wellcome Trust Clinical Research Facility (CRF), Cambridge.

HNR has specialist equipment and facilities for performing nutrition research. The volunteer suite contains a kitchen where all food was prepared and served and a volunteer lounge with facilities for relaxing, watching television, working on the internet and performing activities at a desk, such as crafts. A dining area provides screened off areas for uninterrupted private eating. Interviews, measurements and procedures were conducted in private rooms or cubicles. Cannulations and blood sampling were performed in a separate room dedicated to such procedures. A volunteer manager was available to help with technical difficulties and two medical practitioners and a first aid team were available to assist with complications with the subjects, such as fainting.

The CRF provides facilities for a wide variety of clinical research activities, and can accommodate subjects staying overnight. Two room calorimeters are housed in a separate metabolic research area, together with a room for performing DXA scans and BOD POD measurements. In the studies described in Chapters 4 and 5, subjects stayed overnight at the CRF from the evening prior to day 1 of the study until the morning of day 5.

## 2.2 Measuring Energy Intake

### 2.2.1 Habitual dietary patterns

Habitual diet or recent changes in diet may influence energy consumed in an experimental period. Likewise, underlying attitudes and eating behaviour, such as restrained eating, may affect energy consumption. These factors must be assessed during subject selection and prior to an investigation period, and attempts must be made to reduce their effect. In the studies reported in this thesis all subjects were asked to not make any changes to their habitual diet prior to and during the investigations. In the study reported in Chapter 4 subjects were provided with a meal the night prior to commencement of the study to standardise baseline hunger as far as possible in order to avoid compensatory over-eating on the study day.

#### *2.2.1.1 Identifying subjects who are restrained eaters*

Restrained eating occurs when individuals attempt to suppress their appetite and can occur in people of any weight. Restrained eaters eat more after a preload meal, in comparison to unrestrained eaters who are able to regulate their eating and eat

appropriately less. Additionally, restrained eaters eat more in a distressing situation than when calm (Lowe, 1993). Therefore, ideally restrained eaters should be excluded from short-term investigations involving energy intake.

There are two main questionnaires used to assess restrained eating, the Three Factor Eating Questionnaire (TFEQ) and the 26 item Eating Attitudes Test (EAT-26). Both have been designed to identify eating disorders in a clinical context but have also been used in research involving healthy volunteers to distinguish subjects with abnormal eating. Stunkard *et al.* (1985) proposed the TFEQ to investigate the effect of (1) cognitive restraint of eating, (2) disinhibition and (3) hunger in eating behaviour, particularly in overweight subjects or those with a psychiatric condition (Stunkard & Messick, 1985). They argued that disinhibition was equally important as restrained eating in conditions such as depression as a risk for weight gain, and high scores on the hunger section of the questionnaire predicted binge-eating behaviour in obesity. All three factors were therefore important to include in their questionnaire, particularly when assessing subjects who were healthy and of normal weight (see Appendix II.2.7).

The Eating Attitudes Test (EAT) was first proposed as a 40 item questionnaire to identify symptoms of anorexia nervosa (Garner & Garfinkel, 1979). The abbreviated 26 item questionnaire (EAT-26) has now been used extensively, not only in the diagnosis of anorexia nervosa, but also to identify normal subjects who show a tendency towards restrained eating (Garfinkel & Newman, 2001). Five final behavioural questions are aimed at determining the presence of extreme weight-control behaviours and provide an estimate of their frequency. It is recognised as a test with good reliability and validity,

with sensitivity of 88% and specificity of 96% for abnormal eating (Garfinkel & Newman, 2001). The EAT-26 is simple to perform and was therefore suitable to use as a screening tool in the studies reported in this thesis. Subjects' obtain scores between 0 and 3 for each of the 26 questions, with a maximal score for the questionnaire of 78. Subjects who score above 20 are considered to need further investigation and were therefore excluded from these studies (see Appendix II.2.8).

#### *2.2.1.2 Habitual Diet*

In experimental studies, measuring the subjects' habitual diet is important to identify factors which may alter hunger or fasting blood parameters the following day, such as over- or under-consumption, missing meals, or eating fatty or energy-dense meals. A 24 h recall of food consumption prior to the investigation day can identify whether the subjects' diet may have been affected by any of these factors, and in a within-subject design the diet record may be compared between visits. A 24-h recall was used in the study reported in Chapter 3. Subjects were not scheduled for investigation days close to celebrations that involved feasting or alcohol and the previous day's diet recall was able to identify any unusual eating behaviour.

In the studies reported in Chapters 4 and 5 subjects recorded their habitual diets by writing food diaries, a method that has been used extensively to assess eating patterns in a variety of research settings. Although 7-day weighed food diaries provide the most reliable measure of habitual diet, they are time-consuming and the accuracy of results may suffer from the significant burden required of subjects to weigh and record all food consumed. 4-day food diaries that include 2 weekdays and 2 weekend days are an

acceptable alternative for both researchers and subjects, and household measures provide similar values to weighed food (Bingham *et al.*, 1997). At HNR, food diaries have been developed that allow space for recording all food consumed throughout the day in specific time periods. An estimate of portion size is required for all food consumed, either by recording information from the packaging of the food, or by estimating a small, medium or large portion with assistance from photographs of a number of different foods presented in different portions. Data obtained from the diaries is coded (Price *et al.*, 1995) and entered into an in-house database based on McCance and Widdowson's The Composition of Foods (FSA, 2002) by experienced coders at HNR. The food menu is separated into major food groups and subgroups from which a specific food can be selected and the portion size entered. Specific information about the macronutrient and micronutrient content of the diet can then be obtained from the details of each food held in the database.

### 2.2.2 Measuring food intake

Energy intake during the studies was measured in the metabolic suite by weighing the amount of food provided to subjects, then weighing the amount of food remaining after *ad libitum* consumption. Food consumed could then be converted to nutrients ingested by using food composition tables, as described in section 2.2.2.1. All food for the *ad libitum* meals was presented to the subjects in a similar manner. A large quantity of food was provided in a serving dish and subjects were able to serve their meal onto a separate plate. Subjects were not aware that the food they were consuming was being recorded as they were advised that the studies were designed to investigate metabolic responses to different foods, such as the response of blood parameters (see section 2.7).

#### *2.2.2.1 Formulation and preparation of diets*

I designed and prepared all meals provided in these studies, at HNR, using household ingredients. The composition of the diets was calculated on a specifically designed spreadsheet using data from the McCance and Widdowson tables (FSA, 2002). The composition of foods was taken from the tables and entered into the spreadsheet. Quantities of ingredients were then adjusted to provide the appropriate macronutrient composition. Additional ingredients, such as bran flakes, were added to ensure that factors, such as fibre, were kept as constant as possible. Olive oil was added to the preload meals in Chapter 3 to balance out the fatty acid profile, given the high monounsaturated fat content of the nuts and legumes meal. Egg white or milk powder was used where additional protein was required. Low fat dairy products or tofu were used when the fat content needed to be lowered while keeping the protein content high. Details of additional ingredients and methods used are presented in the chapters describing the studies.

All food was informally tested for taste and consistency prior to the studies commencing, and cooked meals were weighed to accurately assess energy density. In the studies described in Chapters 4 and 5, the water content of the meals was adjusted to keep the energy density as constant as possible for the same meal in spite of different macronutrient composition.

All ingredients were bought in local shops, in large quantities where possible, to reduce the variability of composition that can occur with different batches of produce. If possible, the meals were prepared in advance in the kitchen at HNR and stored in the

freezer. All ingredients were weighed using scales (Sartorius type 1574, GMBH, Gottingen, Germany) which measure to an accuracy of 0.01 g up to 400 g and 0.02 g between 400 and 800 g. Food was prepared and cooked according to study protocols. During the study week, food was defrosted and heated according to standard protocols and the temperature of food about to be served was measured with a temperature probe (Foodcheck, ETI Ltd, Worthing, Sussex, UK). Food was therefore served hot enough to comply with food safety recommendations but did not exceed a safe temperature for consumption (65-80 °C). Fresh food was bought the day before or the day of the study and prepared the day it was to be consumed.

### 2.2.3 Measuring nitrogen excretion

Protein intake can also be estimated by measuring 24-h urine nitrogen excretion, a reliable biomarker used to calculate nitrogen (and hence protein) intake and oxidation rates (Bingham, 2003). 7-day weighed and estimated food diaries show good correlation between recorded protein intake and 24-h urine nitrogen excretion (Bingham, 1997) when subjects are in nitrogen balance, and there is no accumulation due to growth or repair of muscle tissue or loss due to starvation (Bingham, 2003).

In the studies reported in Chapters 4 and 5, urinary nitrogen was measured to verify that the experimental diets were modulating nitrogen excretion and to calculate metabolic protein oxidation. Subjects were confined to the metabolic suite for 4 days prior to the measurement, to ensure they had not experienced any muscle damage, strenuous exercise, excess intake or starvation, which may have interfered with the results.

The reference method for measuring total urinary nitrogen is the Kjeldahl digestion method. The sample is decomposed in hot concentrated sulphuric acid to convert organic nitrogen to ammonium sulphate and oxidize carbon and hydrogen to carbon dioxide and water. Because it is time-consuming and potentially dangerous to laboratory staff, the Kjeldahl method has been replaced in many laboratories by the Dumas method, a safe and accurate alternative, with results about 1.5% higher than with the Kjeldahl method (Thompson *et al.*, 2002). Total nitrogen was determined using this method by the Institute of Grassland and Environmental Research, Aberystwyth, Ceredigion, Wales on a Leco FP 428 nitrogen determinator (Leco Corporation, Michigan, USA). The analysis removes nitrogen by high-temperature combustion and measures it with a thermal conductivity detection system. The between-run coefficients of variation were 0.015%.

## 2.3 Measuring appetite and satiety

Subjective measures of appetite and satiety are critical to understanding the factors that influence energy intake and eating behaviour. It has been argued that measurements of satiety provide a more reliable indicator of the physiological effects of eating (Flint *et al.*, 2000) but a relationship between appetite ratings and subsequent energy intake is not always present (Raben *et al.*, 1995).

The most commonly used method for assessing subjective appetite sensations are visual analogue scale (VAS) scores (Raben *et al.*, 1995). Lines are anchored at each end by words expressing the most positive or negative sensations (e.g. not at all hungry/very hungry). The lines represent a continuum between these extremes, and subjects mark a

point on the line which they feel corresponds to their sensation at the time. Scores are obtained by measuring the distance from the left end of the line to the mark.

Inter-subject variation accounts for >50% of the variance in VAS, meaning that the method is most suitable for within-subject comparisons (Stubbs *et al.*, 2000b). Reproducibility is lowest during fasting measurements and higher for post-prandial measurements, particularly at 4.5 h after a meal when the role of any outlying ratings is reduced (Flint *et al.*, 2000). Reproducible results can be obtained without dietary standardisation prior to the test day. The accuracy of detecting small differences in satiety has also been estimated. Using a paired design, 18 subjects completing the VAS scores are sufficient to detect a 5 mm difference (when using a 100 mm VAS line) in mean score for satiety, hunger and fullness measures, with 90% power. The sample size can be reduced when the study protocol is more tightly controlled, and increases significantly in an unpaired design or when the study design is more variable (Flint *et al.*, 2000). The sample sizes in the studies in this thesis are therefore adequate to detect a significant 5 mm difference in mean score with 80 – 90% power. As the studies were highly controlled within-subject repeated measure designs and VAS have been shown to predict meal initiation and amount eaten, and be sensitive to alterations in diet composition, particularly in an experimental setting (Stubbs *et al.*, 2000b), they are suitable for use in the studies reported in this thesis.

VAS scores are obtained by measuring the distance from the left side of the line to the subject's mark. Comparisons can be performed between the score at critical timepoints

throughout the study, the mean score during time periods, or the AUC of the scores plotted against time (section 2.8).

Palatability was also measured in the study reported in Chapter 4. Standardised questions were used to identify differences in palatability between meals of a similar appearance but with different macronutrient composition in a within-subject design. These questions have also been reported to elicit reliable and reproducible answers in previous experimental studies and so are appropriate for use in this thesis (Flint *et al.*, 2000).

## 2.4 Measuring energy expenditure

Energy expenditure is defined as the metabolic energy expended as mechanical work, biosynthesis or heat and can be measured using a number of different methods. The most precise is measurement by calorimetry involving whole-body measurements in an enclosed chamber.

### 2.4.1 Calorimetry

Direct calorimetry measures energy expenditure as the rate at which heat is lost from the body to the environment. Heat loss includes non-evaporative heat losses (radiation, convection and conduction) and evaporation of water (Murgatroyd *et al.*, 1993a). Heat loss is adjusted by peripheral blood flow and posture but is independent of the metabolic processes that produce the heat, so under resting conditions, direct calorimetry is a very accurate measurement of energy expenditure. The measurement procedure is technically complex and costly and is unable to provide information about the relative contributions

of different substrates to expenditure of energy. It is rarely used to study energy expenditure in humans.

Indirect calorimetry measures energy expenditure as the rate at which heat is produced in the body. Heat production is calculated from rates of respiratory gas exchange (oxygen consumption and carbon dioxide production) that occurs with oxidation of the major substrates: carbohydrate, fat, protein and alcohol. Although indirect calorimetry is expensive to perform and requires a considerable degree of expertise, it is technically easier than direct calorimetry and is the method of choice for measurements of energy expenditure and substrate oxidation (Das & Roberts, 2001).

The two calorimeter chambers used in the studies reported in Chapter 5 have 10.4 m2 floor area and 24 m3 volume. They are comfortable bed-sitting rooms within the CRF with an outside window and a window to the inside research office. The rooms are furnished with a bed, armchair, a desk and upright chair, a television, DVD player, internet computers and an exercycle. A washbasin and camping toilet is provided. A telephone and intercom provide communication to the research staff and the outside world. Food enters the room through an air-lock hatch and urine collected in the room is passed out through a second hatch. Blood samples are taken by the subject placing their arm in a latex sleeve through an air-locked hole in the door.

Air within the rooms was circulated at 20 $m^3$/min, mixing the subject's expired air with room air to produce uniform room air composition. Air temperature was kept at 23 ± 0.5°C. Fresh air to ventilate the rooms was introduced at 200L/min and samples of the

room air were taken at 200 s intervals. Fresh air samples were taken every 20 min, and every two hours calibration checks were performed. The moisture content of the samples was measured (Dewpoint analyser type 1100 ap, General Eastern, Watertown, MA, USA), before analysis of oxygen and carbon dioxide concentrations (paramagnetic O2 analysers types 184 and 1440 and infra-red CO2 analysers type 1510; Servomex, Crowborough, UK). Data was logged onto a computer every 5 min and the room environment could be checked on the computer screen by the research team.

Oxygen consumption and carbon dioxide production was calculated using the expressions of Brown *et al* (Brown *et al.*, 1984). These equations are based on the principle that for any gas at standard temperature and pressure:

Rate of increase of volume of gas inside the chamber

= rate of volume flow of gas into the chamber

+ rate of net volume production of gas by the subject

- rate of volume flow of gas out of the chamber

Corrections are therefore made for temperature and pressure and moisture content and the equations allow for analysis of rapid changes in gaseous exchange between a subject and the environment. The duration that subjects spent in the calorimeter was separated into specific time periods in order to calculate energy expenditure at rest (basal metabolic rate), after meals (diet-induced thermogenesis), during the night (sleeping metabolic rate), and for the entire 24-h period.

Calculation of substrate oxidation rates were performed using the expressions of Murgatroyd *et al* (Murgatroyd *et al.*, 1993b), and the values for respiratory quotients and

energy equivalents of oxygen of Elia and Livesey (Elia & Livesey, 1992). The net oxidation of fat, F, and carbohydrate, C, are expressed in terms of oxygen consumption, $O_2$, carbon dioxide production, $CO_2$, and nitrogen excretion, *N*:

$$\text{Fat} = [CO_2 - R_c\, O_2 + (R_c - R_p)V_p\, k\, N]/V_f(R_f - R_c) \quad (1)$$

$$\text{Carbohydrate} = [CO_2 - R_f\, O_2 + (R_f - R_p)V_p\, k\, N]/V_c(R_c - R_f) \quad (2)$$

where $R_f$, $R_c$ and $R_p$ are the ratios of carbon dioxide production to oxygen consumption and $V_f$, $V_c$, and $V_p$ are the volumes of oxygen consumed per gram oxidized for fat, carbohydrate and protein respectively. The constant, *k*, relates protein oxidation to nitrogen excretion, the protein oxidation rate being therefore *k* N. The values for the constants used in this study are $R_c = 1.0$, $R_f = 0.71$, $R_p = 0.835$. $V_c = 0.746$ L/g, $V_f = 2.01$ L/g, $V_p = 0.952$ L/g, and $k = 6.25$ (Elia & Livesey, 1992).

When these values are inserted into the equations the results are:

$$\text{Fat} = -1.7156[CO_2 - O_2 + 0.9818.N]$$

$$\text{Carbohydrate} = 4.6224[CO2 - 0.71\ O2 - 0.7437\ N]$$

$$\text{Protein} = 6.25\ N$$

where fat, carbohydrate and protein are measured in grams when oxygen and carbon dioxide are in litres and nitrogen is in grams.

### 2.4.2 Portable indirect calorimetry

Portable measures of energy expenditure use the principles of indirect calorimetry to measure the concentration differences of oxygen and carbon dioxide between inspired and expired air. A ventilated canopy is placed over the subject's head. A constant flow of

air (between 20 and 80 L/min) enters the canopy and expired air is drawn through a mixing chamber and passed through sensors that measure $O_2$ and $CO_2$. Calculations can then be performed to measure resting energy expenditure. Due to the small residual air volumes (in comparison to a whole-body chamber), measurements of energy expenditure using this method may be more precise than classical room calorimetry, although measurements can be affected by air quality in the room or by subject movement during the measuring period. Measurements are limited to resting energy expenditure (basal metabolic rate) due to the requirement that subjects must lie still prior to and during the procedure. The cost of performing this procedure is significantly lower than that of whole-body calorimetry, and together with the short time frame to complete a measurement (20 – 30 min), portable indirect calorimetry provides a useful tool to measure resting energy expenditure when whole-body calorimetry is unavailable or impractical.

In the study reported in Chapter 5, resting oxygen consumption and carbon dioxide production, measured by using a ventilated-hood indirect calorimetry system (GEM Nutrition Ltd. Daresbury, Cheshire) was used to calculate resting energy expenditure at the start and end of each study week. The results from the first study day were used to calculate a predicted total energy expenditure, which provided the basis for the energy content of the meals prepared for each subject during the fixed energy intake day in the calorimeter. Further details are reported in Chapter 5.

## 2.5 Measuring body composition

The variation in body composition between individuals is large, mainly due, in healthy, active young subjects, to differences in fat mass. Excess body fat, especially abdominal adiposity is an important indicator of the metabolic syndrome and a contributor to the risk for metabolic and cardiovascular diseases.

### 2.5.1 Measuring whole body adiposity

In order to measure whole body adiposity accurately a number of different components of body composition can be identified. The simplest model assumes that the body is composed of two compartments, fat and fat-free mass. This is the basis for the whole body density method, or estimates based on total body water. Total body water can be measured by isotopically labelled tracer and, by combining density and body water, it is possible to derive a three component model – water, fat and dry fat free mass. Using techniques such as DXA to measure bone mineral allow for further separation of fat-free mass into minerals and protein. Body composition can therefore be separated into the four compartments: fat mass, total body water, minerals and protein (Jebb & Elia, 1993) (Deurenberg & Roubenoff, 2002).

#### *2.5.1.1 Whole-body density*

Underwater weighing has been the gold-standard method for measuring body density and hence body fat mass (Deurenberg & Roubenoff, 2002). The weight of a subject is measured in air and then while fully submerged underwater. The difference between the weight in air and the weight underwater calculates the body volume, once residual lung

volume (measured) and air in the gut (assumed to be 100 ml for a fasted subject) have been subtracted (Jebb & Elia, 1993). This technique is very accurate and provides reproducible results but is limited by the ability of subjects to tolerate the procedure.

An alternative to measuring body volume underwater is to measure by air displacement. In the Bod Pod (Life Measurements, Inc, Concord, CA) body volume is measured in a sealed machine containing two chambers, separated by a diaphragm, with a known volume of air. The subject sits in the front chamber with the door closed and the change in pressure between the front and rear chambers is measured, from which the body volume is calculated. A predicted lung volume is used to calculate body density using the formula:

Body density = Mass/$Vb_{raw}$ + 0.40 – SAA + 40% $V_{TG}$

where $Vb_{raw}$ = raw body volume, SAA = surface area artifact and $V_{TG}$ = thoracic gas volume (Ball & Altena, 2004). Good agreement has been found between underwater weighing and air displacement, with air displacement being better accepted by subjects (Deurenberg & Roubenoff, 2002).

Both methods assume that the body is composed of two compartments, fat and fat-free mass, each with a constant density, 0.9 kg/L for fat and 1.1 kg/L for fat-free mass. Density is measured as the mass of the body in air divided by the volume of body tissues (Jebb & Elia, 1993). Once the density of the body has been determined the proportion of fat in the body can be calculated using Siri's equation:

% fat = [495/body density] – 450] * 100

Fat and fat-free mass were measured in the studies reported in Chapters 4 and 5 by the Bod Pod, as the most tolerable procedure to measure whole body density. A standard protocol was followed for each measurement. The Bod Pod was turned on and warmed up for 30 min, then calibrated using a 50 L cylinder. An auto run was performed to ensure the accuracy of the machine following calibration. Subjects were fasted and tested immediately after resting (after the measurement of BMR by the GEM portable calorimeter). Subjects were dressed in tight fitting swimwear or underwear and a lycra swimming cap, and all jewellery and watches were removed. Subjects entered the Bod Pod and were asked to sit quietly without leaning against the back wall to prevent air trapping. They were shown a green button which illuminates when the door is held closed by the magnetic latches and can be used in an emergency to release the door. Two measurements of body volume were taken and a third measurement was performed if the first two differed by more than 150 ml. Subjects were seated in the Bod Pod for approximately 5 min while the measurements took place. Once completed the door was opened and the subjects able to leave.

#### *2.5.1.2 Dual energy x-ray absorptiometry*

Dual energy X-ray absorptiometry (DXA) measures three components of the body, fat mass, lean mass and bone mineral (Jebb & Elia, 1993). The body is scanned with X-rays at two distinct levels of energy (40 keV and 70 keV) (Pietrobelli *et al.*, 1996). The differential attenuation of the tissues for these two levels of radiation depends on its chemical composition. Software in the DXA scanning machines uses specific algorithms to distinguish bone mineral from soft tissue, and subsequently divides soft tissue into fat and lean tissue. Identification of fat mass, lean mass and bone mineral are therefore

possible in different areas of the body, legs, trunk, spine, femur and arms, but DXA scanning cannot differentiate subcutaneous adipose tissue from internal adipose tissue depots (Deurenberg & Roubenoff, 2002).

DXA scanning involves only a small amount of radiation, particularly as newer technology reduces scanning time. For the whole-body scans used in this thesis, the radiation dose per scan was <0.4 μSv which is equivalent to less than one sixth of the background radiation dose received in one day.

A limitation in the use of DXA scanning is that attenuation of the X-rays depends on the thickness of the tissue, so that correction for body size must be made. Subjects must also fit within the scanning area which may be difficult for the exceptionally tall or obese. Some parts of the body of such subjects are left out of the scanning image; the calculations must be adjusted for these omissions (Jebb & Elia, 1993) (Deurenberg & Roubenoff, 2002). However, as subjects just need to lie still on the scanning bed, reproducibility of DXA is very high (between 0.5% to 2%).

In this thesis subjects were scanned using a Lunar Prodigy Advance (GE Medical Systems LTD, UK). Standard DXA quality control and calibration measures were performed prior to each testing session. Subjects were scanned in the fasted state after the Bod Pod measurements. Subjects were asked to remove shoes and all metal objects such as watches and jewellery. Subjects were then asked to lie on the DXA scanner table and were positioned accurately for the scanning beam and to ensure that the position was the same for every scan. When the scan started, the scanning arm moved over the subject to

obtain readings for the attenuation of tissue. Subjects were asked to lie still, while breathing normally. Scanning took 5 to 10 minutes and the scan was analysed to determine fat mass, lean mass and bone mass.

### 2.5.2 Measuring abdominal adiposity

Imaging techniques enable an accurate assessment of individual organs, location and quantity of adipose tissue, and detailed imaging of muscle mass.

Computed tomography (CT) has provided the gold standard in imaging techniques, particularly to distinguish visceral fat from subcutaneous fat. During CT scanning, a source of X-rays rotates around the part of the body to be examined. Photodetectors on the opposite side of the source of X-rays detect the attenuation of the X-rays after they have passed through the body. Images of the tissues present in each cross-sectional slice are then generated. CT scanning is very accurate, with an error of about 1%, and the scan is relatively fast. However, the cost, the need for the output to be read by a trained radiologist, and the significant level of radiation exposure during a scan limits its widespread use (Deurenberg & Roubenoff, 2002).

Magnetic resonance imaging (MRI) does not involve radiation and is becoming increasingly used to accurately measure visceral fat (Gomi *et al.*, 2005). A subject enters a cylindrical chamber which contains a strong magnetic field. Nuclei with intrinsic magnetic properties align themselves in the direction of the magnetic field when a radio-frequency wave is passed through the body. When the radiowave is turned off the nuclei relax and emit energy that they had absorbed (Jebb & Elia, 1993). The signals emitted are

used to generate an image of each cross-section, and accurate information about the tissues present at each slice is obtained. MRI scanning is more expensive than CT scanning and the time taken to complete a scan is considerably longer, making MRI impractical for general use.

Recently a new instrument has been developed to measure body composition. Quantitative magnetic resonance (QMR), EchoMRI uses similar principles to MRI. The differences in nuclear magnetic resonance properties of hydrogen atoms are used to determine fat mass, lean mass and free water. Precision has been reported to be as small as 0.25 – 0.5 kg and as a scan can be performed in less than 3 minutes this method may become the new standard for measuring differences in fat and lean mass (Napolitano *et al.*, 2008).

The above methods are expensive and time-consuming, and in the case of CT scanning involve a significant degree of radiation exposure. A useful proxy to indicate abdominal adiposity is to measure waist circumference (Lean *et al.*, 1995) (Browning *et al.*, 2010). A measurement at the mid-point between the lower rib and the superior border of the iliac crest measures waist circumference to the nearest 0.5 cm. Measurements are complicated by differences in investigator technique, and the bony 'landmarks' being difficult to locate, particularly in the obese.

## 2.6 Assessing metabolic risk

### 2.6.1 Body mass, height and BMI

All measurements were made after an overnight fast.

Weight was measured to the nearest 0.1 kg using Seca digital scales (Seca GB, Birmingham, UK), with subjects wearing light indoor clothing, and no shoes. Height was measured to the nearest 0.1 cm using a wall-mounted Seca measuring rod (Seca GB, Birmingham, UK). Subjects stood upright without shoes, with knees and back straight. Repeated measurements of height and weight over the course of the study were made using the same scales and measuring rod on each occasion.

Body mass index (BMI) was calculated by the following equation:

$$\text{BMI} = \frac{\text{Weight (kg)}}{\text{Height (m)}^2}$$

### 2.6.2 Blood pressure

Blood pressure was measured using an automated Dinamap blood pressure monitor (Critikon, Dinamap PRO200 monitor, Tampa, FL, USA). Subjects were rested in the seated position for 5 minutes and three measurements were taken at 2 min intervals. The first measurement was discarded and a mean was calculated for the second and third readings.

### 2.6.3 Blood markers of satiety and metabolic risk

Blood markers of satiety and metabolic risk, indicating presence or future risk of the metabolic syndrome, were recorded from venous blood samples during the studies reported in this thesis. In the study reported in Chapter 3, glucose, insulin, and non-esterified fatty acids (NEFA) concentrations were measured from fasting and post-prandial blood samples, while in the studies in Chapter 4 and 5, fasting blood samples were taken for measurement of glucose, insulin, total cholesterol, triacylglycerol, LDL cholesterol, HDL cholesterol, and high sensitivity C-reactive protein (hsCRP), and post-prandial samples for measurement of glucose, insulin, ghrelin, GLP-1, PYY, PP, insulin-like growth factor 1 (IGF-I) and insulin-like growth factor binding protein 3 (IGF-BP3).

Fasting blood samples were taken after an overnight fast during which time subjects were allowed to only drink water. Post-prandial samples were taken at specific timepoints as detailed in the protocol for each study. Samples were taken though an 18 or 20 gauge intravenous cannula (Vygon biovalve cannula, Laboratory Pharmaceutiques, Ecouven, France) inserted into a large vein in the antecubital fossa or the forearm. After insertion, the first blood samples were taken and the cannula was flushed with 5 – 10 ml of normal saline. The cannula was secured and subjects were asked to avoiding bending their arm if the cannula crossed the elbow joint. At each blood-sampling timepoint, 2 ml was removed and discarded prior to taking the sample. The cannula was then flushed with normal saline. If problems occurred with sampling 0.5 ml of heparin (Canusal heparin sodium (mucous) BP. 100 units/ml, CP Pharmaceuticals Ltd, Wrexham, UK) was flushed into the cannula and left until the next sampling period.

Samples were aliquotted into monovettes (Sarstedt, Leicester, UK), which were pre-treated with lithium heparin, fluoride, citrate, aprotinin, or a serum gel clotting activator as required. Samples were immediately placed on ice, with serum samples being allowed to coagulate first. All samples were centrifuged for 20 min at 3000 rpm at 4 °C within 1 hour of collection. Plasma and serum samples were then aliquotted into pre-labelled 2 ml microtubes (Sarstedt Ltd., Leicester, UK) and stored at –80 °C until analysis.

All human samples collected during this study were processed at HNR or at the CRF, in a sample-processing laboratory equipped with centrifuges, class II safety cabinets and a urine processing cabinet.

The assays used and methods of analysis are described below. One limitation of these analyses is that many of the data were close to the limit of detection, defined as the lowest quantity of a substance that can be distinguished from the absence of that substance. This limit may however be lower than the limit of quantification, the level where the analysis precision allows for reasonable detection of the difference between two different values. The values close to the limit of detection may have occurred because samples were taken from healthy subjects with subtle changes in conditions which produced relatively small changes in blood analytes.

#### *2.6.3.1 Glucose*

Plasma samples were collected in monovettes pre-treated with fluoride (Starstedt, Leicester, UK). Samples were analysed by the Nutritional Biochemistry Laboratory (NBL) at HNR on the Siemens ARx clinical chemistry system (Frimley, Camberley,

Surrey GU16 8QD). The method is an adaptation of the hexokinase-glucose-6-phosphate dehydrogenase method which is the generally accepted reference method for measuring glucose. Hexokinase (HK) catalyzes the phosphorylation of glucose in the presence of adenosine-5'-triphosphate (ATP) and magnesium to form glucose-6-phosphate (G-6-P) and adenosinediphosphate (ADP). G-6-P is then oxidized by glucose-6-phosphate dehydrogenase (G-6-PDH) in the presence of nicotinamide adenine dinucleotide (NAD) to produce 6-phosphogluconate and NADH. One mole of NAD is reduced to one mole of NADH for each mole of glucose present. The absorbance due to NADH (and thus the glucose concentration) is determined by photometry using a bichromatic (light at wavelengths 340 and 383 nm) endpoint technique.

All reagents, standards and consumables were those recommended and supplied by the manufacturer. The assay range was 0 - 27.8 mmol/l and between run coefficients of variation were 2.6% at 3.24 mmol/l, 2.8% at 6.73mmol/l and 2.3% at 19.84 mmol/l.

### *2.6.3.2 Continuous glucose monitoring*

In the studies reported in Chapters 4 and 5 interstitial glucose was measured over two 24 h periods. Although changes in interstitial glucose lag behind those of blood glucose, the frequency of measurement (at 5 min intervals) allows for a more detailed investigation of glucose metabolism in response to changes in diet composition throughout the day.

When fitted with the continuous glucose monitoring system (CGMS (Gold) Continuous Glucose Monitoring System, Medtronic, UK) subjects were given full training in how to operate it. The sensor was inserted according to the manufacturer's instructions, using the insertion device provided, and employing sterile technique. The insertion site was

selected as an area with adequate subcutaneous fat, most frequently the lower abdomen, avoiding the belt/waistline or other areas where clothing could rub or constrict, the 5 cm area around the navel, and any scarred or atrophied tissue. Subjects were instructed in how to take a finger-prick blood glucose reading using the Accu-chek Advantage system (Roche Diagnostics Limited, Lewes, UK). A lancet is inserted into a pen device and with the device held firmly against the skin a button is pressed to release the lancet and prick the skin. When a small drop of blood is obtained it is held up to a testing strip that has been inserted into the meter. Blood is automatically drawn up into the test strip and the meter begins to analyse the glucose measurement. Subjects were instructed in how to enter readings into the CGMS monitor and asked to enter 4 readings per day for calibration of the monitor, at any time excluding the half hour following eating. Subjects were also instructed in how to enter 'events' (i.e. food, exercise), and possible reasons for and what to do if the monitor alarmed. Subjects were given a simple instruction leaflet (Appendix II.2.10) and provided with 24-hour phone numbers for contacting both the chief investigator and a representative from Medtronic in case of difficulties.

#### *2.6.3.3 Insulin*

Plasma samples were collected in monovettes pre-treated with lithium heparin (Starstedt, Leicester, UK). Samples were analysed by the Clinical Biochemistry Department at Addenbrooke's Hospital, Cambridge, UK, on a 1235 AutoDELFIA automatic immunoassay system using a two-step time resolved fluorometric assay (Kit No. B080-101). All reagents, standards and consumables were those recommended by the manufacturer. The calibrators were referenced to WHO 1st IRP 66/304.

Cross-reactivity with intact pro-insulin is < 0.5 % at 2736 pmol/l, 32-33 split pro-insulin 1% at 2800 pmol/l, C-peptide <0.1% at 5280 pmol/l. The limit of detection is 1.3 pmol/l, and the assay range without dilution is approximately 1100 pmol/l. Between batch coefficients of variation were 3.1% at 29 pmol/l, 2.1% at 79.4 pmol/l, 1.9% at 277 pmol/l & 2.0% at 705 pmol/l (n = 174).

*2.6.3.4 Ghrelin*

Plasma samples were collected in monovettes pre-treated with EDTA (ethylenediaminetetraacetic acid) as an anticoagulant and aprotinin as an enzyme inhibitor (Starstedt, Leicester, UK). Samples were acidified prior to analysis at the Centre for Diabetes and Endocrinology, University College London. Active ghrelin was measured by Sandwich ELISA, which involves capture of the active form of human ghrelin molecules in the sample by anti-human ghrelin IgG and the simultaneous binding of a second antibody to ghrelin. Unbound material is washed away and the bound enzyme is conjugated with horseradish peroxidase. The activity of the antibody-enzyme conjugate is measured spectrophotometrically by increased absorbency at 450 nm. Since the increase in absorbency is directly proportional to the amount of captured active human ghrelin in the sample, the concentration of active ghrelin can be derived from a reference curve generated in the same assay with reference standards of known concentrations of human ghrelin. All reagents, standards and consumables were those recommended by the manufacturer.

Sensitivity of the assay at the 95% confidence limit was 25 pg/ml and the inter-assay coefficients of variation were 3.6% at 65.2 pg/ml and 9.3% at 397 pg/ml.

*2.6.3.5 Glucagon-like peptide-1 (GLP-1)*

Plasma samples were collected in monovettes pre-treated with EDTA (ethylenediaminetetraacetic acid) as an anticoagulant and aprotinin as an enzyme inhibitor (Starstedt, Leicester, UK). Samples were analysed at the Centre for Diabetes and Endocrinology, University College London. Active GLP-1, (7-36 and 7-37 amides) was measured by an ELISA assay which captures active GLP-1 from the sample by a monoclonal antibody, immobilized in the wells of a microwell plate, that binds specifically to the N-terminal region of the active GLP-1 molecule. Unbound material is removed and added anti GLP-1 alkaline phosphatase conjugates to the immobilised GLP-1. The bound conjugate is detected by adding MUP (methyl umbelliferyl phosphate) which in the presence of alkaline phosphatase forms the fluorescent product umbelliferone. Since the amount of fluorescence generated is directly proportional to the concentration of active GLP-1 in the sample, the concentration of active GLP-1 can be derived from a reference curve generated in the same assay with reference standards of known concentrations of active GLP-1. All reagents, standards and consumables were those recommended by the manufacturer.

Sensitivity of the assay at the 95% confidence limit was 2.0 pM and the inter-assay coefficients of variation were 13% at 4 pM and <1% at 76 pM.

*2.6.3.6 Peptide tyrosine tyrosine (PYY)*

Plasma samples were collected in monovettes pre-treated with EDTA (ethylenediaminetetraacetic acid) as an anticoagulant and aprotinin as an enzyme inhibitor (Starstedt, Leicester, UK). Samples were analysed at the Centre for Diabetes

and Endocrinology, University College London. Total PYY was measured by an enzyme immunoassay using an antibody to PYY and a labelled antigen. Horse radish peroxidase (HRP) labelled streptoavidin (SA) is added to form HRP labelled streptoavidin-biotinylated antigen-antibody complex. HRP enzyme activity is determined by 3,3',5,5'-Tetramethylbenzidine (TMB) and the concentration of human PYY is calculated. All reagents, standards and consumables were those recommended by the manufacturer.

Sensitivity of the assay at the 95% confidence limit was 1.4 pg/ml and the inter -assay coefficients of variation were 5.45-10.26%

*2.6.3.7 Pancreatic polypeptide (PP)*

Plasma samples were collected in monovettes pre-treated with lithium heparin (Starstedt, Leicester, UK). Samples were analysed at the Centre for Diabetes and Endocrinology, University College London. PP was measured by an ELISA assay by adding the samples to anti-human PP polyclonal antibody and then adding a second antibody to the bound sample. Added horseradish peroxidase conjugates to the bound antibodies and the enzyme activity is measured by quantifying the antibody-enzyme conjugate spectrophotometrically by the increased absorbency at 450 nm. Since the increase in absorbency is directly proportional to the amount of captured human PP in the sample, the latter can be derived from a reference curve generated in the same assay with reference standards of known concentrations of human PP. All reagents, standards and consumables were those recommended by the manufacturer.

Sensitivity of the assay at the 95% confidence limit was 12.3 pg/ml and the inter-assay coefficients of variation were 9.8% at 103 pg/ml and 4.9% at 128 pg/ml.

### *2.6.3.8 Non-esterified fatty acids (NEFA)*

Plasma samples were collected in monovettes pre-treated with lithium heparin (Starstedt, Leicester, UK). Samples were analysed by the NBL at HNR using the Cobas FARA (Roche Diagnostic Ltd., Lewes, UK) using Roche reagents. The assay uses a standard enzymatic method based on acyl-CoA synthetase, acyl-CoA oxidase and peroxidase reactions. The between run total coefficients of variation were 3.1% at 1574.17 μmol/l, and 4.0% at 630.4 μmol/l.

### *2.6.3.9 High-sensitivity C reactive protein (HsCRP)*

Serum samples were collected in monovettes pre-treated with a serum gel clotting factor (Starstedt, Leicester, UK). Samples were analysed by the NBL at HNR using the Siemens BN ProSpec (Siemens, Frimley, Camberley, Surrey GU16 8QD). Polystyrene particles coated with monoclonal antibodies specific to human CRP are aggregated when mixed with samples containing CRP. These aggregates scatter a beam of light passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The result is evaluated by comparison with a standard of known concentration.

The sensitivity of the assay was 0.175 mg/l and the between-run coefficients of variation were 3.1% at 0.69 mg/l, and 3.5% at 49.5 mg/l.

*2.6.3.10 Total Cholesterol*

Serum samples were collected in monovettes pre-treated with a serum gel clotting factor (Starstedt, Leicester, UK). Samples were analysed by the NBL at HNR using the Dimension Xpand Clinical Chemistry Analyser (Siemens, Frimley, Camberley, Surrey GU16 8QD).

Cholesterol esterase catalyses the hydrolysis of cholesterol esters to produce free cholesterol which, along with pre-existing free cholesterol, is oxidized in a reaction catalysed by cholesterol oxidase to form cholest-4-en-3-one and hydrogen peroxide. In the presence of horseradish peroxidase, the hydrogen peroxidase formed is used to oxidize N,N-diethylaniline-HCL/aminoantipyrine to produce a chromophore. The absorbance of this chromophore is proportional to the total cholesterol concentration and is measured using a polychromatic endpoint technique.

The assay range is 1.3-15.5 mmol/l and between-run total coefficients of variation were 3.8% at 2.41 mmol/l, 2.5% at 4.05 mmol/l and 1.6% at 6.43 mmol/l.

Samples with higher apparent concentrations were re-assayed following dilution in purified water. Samples with apparent concentrations below the assay range were diluted 1:1 in a high concentration QC material, re-assayed and if confirmed reported as <1.3 mmol/l.

*2.6.3.11 Triacylglycerols*

Serum samples were collected in monovettes pre-treated with a serum gel clotting factor (Starstedt, Leicester, UK). Samples were analysed by the NBL at HNR using the Dimension Xpand Clinical Chemistry Analyser (Siemens, Frimley, Camberley, Surrey GU16 8QD).

An enzymatic assay was used to measure the serum or plasma concentrations of triacylglycerol. The samples are incubated with lipoprotein lipase (LPL) that converts triacylglycerols into glycerol and fatty acids. Glycerol kinase (GK) catalyzes the phosphorylation of glycerol by adenosine-5-triphosphate (ATP) to glycerol-3-phosphate. Glycerol-3-phosphate-oxidase oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate and hydrogen peroxide ($H_2O_2$). The catalytic action of peroxidase (POD) forms quinoneimine from $H_2O_2$, aminoantipyrine and 4-chlorophenol. The change in absorbance due to the formation of quinoneimine is directly proportional to the total amount of glycerol and its precursors in the sample and is measured by photometry using a bichromatic (510, 700nm) endpoint technique.

The assay range is 0.17 – 11.3 mmol/l and between run coefficients of variation were 7.0% at 0.847 mmol/l, 5.9% at 1.315 mmol/l, and 3.6% at 2.111 mmol/l.

*2.6.3.12 High-density lipoprotein (HDL)*

Serum samples were collected in monovettes pre-treated with a serum gel clotting factor (Starstedt, Leicester, UK). Samples were analysed by the NBL at HNR using the

Dimension Xpand Clinical Chemistry Analyser (Siemens, Frimley, Camberley, Surrey GU16 8QD).

The automated HDL assay is a homogeneous method for directly measuring HDL cholesterol levels without the need for off-line pre-treatment or centrifugation steps. The reference method for the quantitation of HDL cholesterol combines ultracentrifugation and chemical precipitation to separate HDL from other lipoproteins, followed by cholesterol measurement using the Abell-Kendall assay. This method is too time consuming and labour intensive for use in routine analysis. Therefore most laboratories use an alternative method involving selective precipitation and removal of LDL and VLDL, followed by the enzymatic assay of HDL cholesterol in the supernatant fraction. A specific detergent selectively solubilises HDL and a standard enzymatic assay based on cholesterol oxidase and peroxidase reactions is used.

The assay range is 0.26 - 3.89 mmol/l and between run coefficients of variation were 7.5% at 0.93 mmol/l, 5.2% at 1.58 mmol/l, and 3.6% at 2.03 mmol/l.

*2.6.3.13 Low-density lipoprotein (LDL)*

LDL cholesterol was calculated from values for total cholesterol, HDL cholesterol and triacylglycerols using Friedewald's formula (Friedewald *et al.*, 1972):

LDL cholesterol = [Total cholesterol] - [HDL- cholesterol] - ([triacylglycerol]/2.2)

LDL cannot be calculated if the triacylglycerols are > 4.52 mmol/l.

*2.6.3.14 Insulin-like growth factor-1 (IGF-I)*

Serum samples were collected in monovettes pre-treated with a serum gel clotting factor (Starstedt, Leicester, UK). Samples were analysed by the Nutrition Bone Health Laboratory at HNR using the Immulite 1000 system.

Immulite 1000 IGF-I is a solid phase, enzyme-labelled chemiluminescent immunometric assay, which has 60 min incubation cycle. Because of the presence of acid-labile components and binding proteins, samples are pre-treated with acid to release IGF-I.

The sensitivity of the assay was 20 ng/ml and the intra-assay coefficients of variation were 6.1% at 49 ng/ml and 5.8% at 955 ng/ml.

*2.6.3.15 Insulin-like growth factor binding protein 3 (IGF-BP3)*

Serum samples were collected in monovettes pre-treated with a serum gel clotting factor (Starstedt, Leicester, UK). Samples were analysed by the Nutrition Bone Health Laboratory at HNR using the Immulite 1000 system.

Immulite 1000 IGFBP-3 is a solid phase, enzyme-labelled chemiluminescent immunometric assay, which has 30 min incubation cycle.

The sensitivity of the assay was 0.1 $\mu$g/ml and the intra-assay coefficients of variation were 9.1% at 0.55 $\mu$g/ml and 8.5% at 7.8 $\mu$g/ml.

## 2.7 Nature of the research

These studies were performed according to the British Sociological Association recommendations for covert research (BSO, 2002). Subjects were informed that they were participating in studies to investigate the 'Metabolic response to different meal types' for the study reported in Chapter 3 (see Appendix I.2.1) and investigating 'Diet and energy metabolism' for the studies reported in Chapters 4 and 5 (see Appendix II.2.1). As energy intake, one of the main outcome measures in these studies, was dependent on the subjects' *ad libitum* food consumption, it was important that subjects were not aware that their food intake was being recorded. By attempting to focus on other aspects of the study, such as blood sample analysis, rather than energy intake, the physiological effect of the diets could more precisely be measured independent of psychosocial and environmental eating triggers. The use of covert research is recognised to present ethical and legal issues in conducting research, particularly with the ability of subjects to provide informed consent. It is, however, acknowledged that difficulties arise in research when participants change their behaviour because they know what is being studied, and in this situation covert research is justified.

## 2.8 Statistical analysis

All statistical analyses were carried out using Microsoft Excel 2000 (Microsoft Corporation, USA) and Stata version 9.1 (StataCorp LP, Texas, USA). Normality of distribution of variables was checked using quantile plots, and data were transformed to achieve normality where necessary. Endpoint outcome measures were compared within-person using a random effects model for continuous normal data. Subjects were the only

random effect; to make the analysis within-subject, the fixed effects were the type of protein (in Chapter 3) and percentage of protein (in Chapters 4 and 5). Additionally, in chapter 4, a number of exploratory secondary outcomes were measured. Graphs of endpoints were examined by randomisation group to give an indication of any differing effects by treatment order.

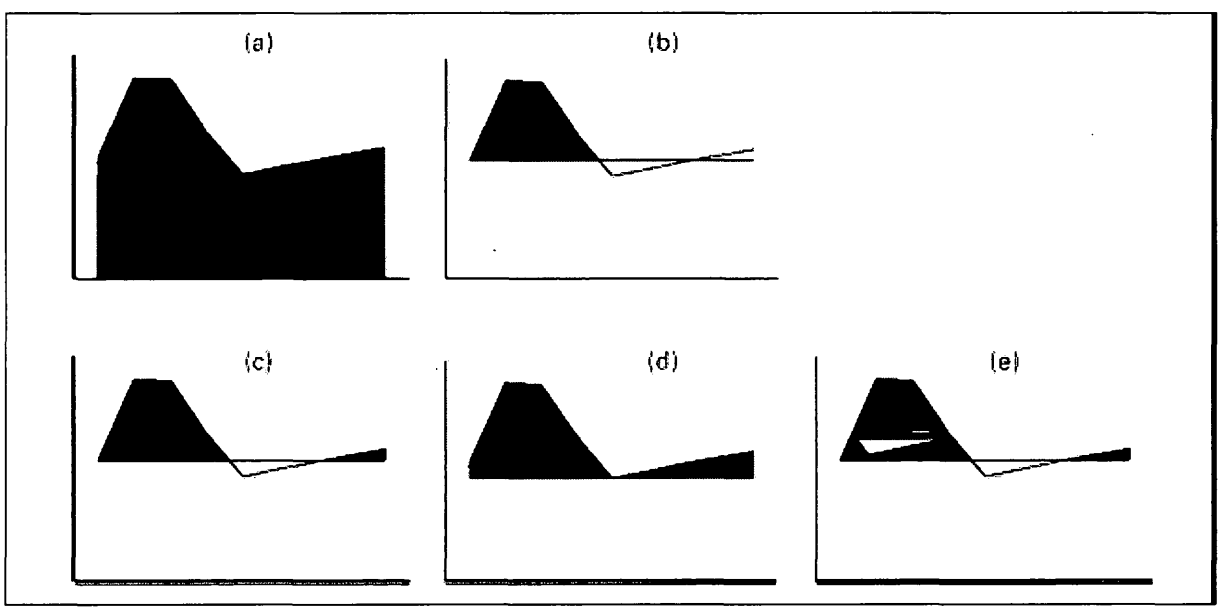
The area under the curve (AUC) was calculated for a post-prandial blood tests and visual analogue scales. The AUC is obtained by applying the trapezoid rule: two adjacent scores are added together and divided by the time between when the scores were recorded. The values for each pair of scores are then added together to obtain the total AUC.

As post-prandial glucose levels can fall below the baseline several methods have been used to calculate the AUC of glucose (Figure 2.8.1) with little consensus about the ideal method (Brouns, 2005). Method 1 (Figure 2.8.1(a)) calculates the total AUC down to a blood concentration of 0. Since the area above the baseline is only a small proportion of total AUC, this method is less sensitive for distinguishing differences in glucose excursions from baseline. Methods 2 – 5 calculate the AUC from fasting to the first return to baseline and then calculate the area under the baseline curve in a number of different ways: by not calculating blood glucose values after the first return to baseline (method 2, Figure 2.8.1(b)), by only calculating values greater than the baseline levels (method 3 Figure 2.8.1(c)), by using the lowest glucose concentration, rather than the fasting concentration as the baseline (method 4 Figure 2.8.1(d)), or by subtracting the area below the baseline from the AUC above the baseline (method 5 Figure 2.8.1(e)). Although the Food and Agriculture Organization (1998) recommend the use of method 3 to measure glycaemic index (Brouns, 2005) this method is not necessarily ideal for investigating

glucose excursions after mixed meals. Glucose concentrations falling below the baseline may be associated with increased hunger and a subsequent increase in energy intake. A flatter profile may therefore be more beneficial when assessing the effect of food on post-prandial glucose levels. In order to include the whole area of the glucose curve and to identify whether any differences were obtained when the area below baseline was subtracted from the total area, the AUC of all glucose curves in this thesis were calculated using methods 1 and 5.

**Figure 2.8.1 Different methods to determine area under the curve (AUC)**

(a) Total AUC, method 1; (b) incremental AUCcut, method 2; (c) incremental AUC, method 3; (d), incremental AUCmin, method 4; (e) net incremental AUC, method 5. From (Brouns, 2005).



# 3 Chapter 3 The effect of protein type on short-term appetite and energy intake

## 3.1 Introduction

Protein, fat and carbohydrate exert differential effects on appetite and satiety, as discussed in Chapter 1. Increasing fat in the diet results in less suppression of hunger and greater energy intake. Dietary recommendations to reduce energy intake have therefore concentrated on reducing fat by increasing carbohydrate. However, protein appears to produce the greatest satiety and may enhance weight regulation. There has been relatively little attention given to the type of protein and, as different protein sources are associated with very different dietary patterns, it is important to refine the basic evidence linking protein to post-prandial satiety to inform dietary recommendations and public health strategies.

### 3.1.1 Methodological approaches

The challenge presented when investigating complex physiological processes, such as appetite and satiety, that are influenced to a large extent by the environment, is whether to replicate habitual conditions as closely as possible, or to provide a high level of control and focus specifically on a few physiological factors. Dietary surveys can ascertain habitual diet in free-living subjects and, when compared to body mass, provide an

indicator of the effect of diet patterns on energy intake. Inferences about the satiety effects of a particular diet can then be made. Surveys provide valuable information about dietary effects in large populations but dietary reporting can be imprecise and complicated by confounding factors, limiting their use in small studies.

Questionnaires to assess appetite have been used in long-term intervention studies where the diet is well-controlled (by providing some or all of the study diet) or closely followed (such as through regular diet counselling sessions). The information obtained reflects satiety in an habitual living environment with moderate control over the diet being consumed, but imprecision and uncertainty about the exact diet and activity of the subjects remain.

Experimental research has frequently used preload studies to investigate the effect of single meals on hunger and satiety. A preload meal of fixed-energy content is provided to subjects, usually after a period of fasting, and subjects are required to consume the entire meal. Measurements of components of satiety follow, and may include visual analogue scales (as discussed in Chapter 2) and blood tests. After a fixed period of time, a second test meal is provided, usually an *ad libitum* buffet style meal in which the energy content of all the dishes is known. Subjects select the quantity of food they wish and are asked to eat until they feel comfortably full. The food selected is weighed before and after the meal to determine the weight of the food consumed, from which the energy of food ingested can be calculated. This objective measure of energy intake is taken as an index of 'hunger' or as a measure of the satiation induced by the preceding meal.

Preload studies are highly controlled and therefore provide a valuable tool to investigate post-prandial properties of food. However, the experimental nature of the study design, with meals eaten and measurements performed in a metabolic suite, limit the translation of preload studies to a free-living eating environment.

Preload studies have been used to investigate the effect of macronutrients on satiety and energy intake (e.g. (Stubbs *et al.*, 1996), (Poppitt *et al.*, 1998), and the properties of macronutrients such as the glycaemic index of carbohydrates (Warren *et al.*, 2003) and fatty acid composition of food (Westerterp-Plantenga, 2004), (Flint *et al.*, 2003). Only a few studies have investigated the effect of protein type on satiety and energy intake.

Comparisons between preload studies are difficult because of variations in subject characteristics and study design. Studies may be performed on lean or obese subjects. Bowen *et al* (2006) argue that strategies to optimise satiety are most applicable to overweight subjects as a means of improving compliance with energy restricted diets (Bowen *et al.*, 2006c), however studies of only the overweight or obese may not be relevant to a lean population or for the prevention of overweight and obesity. Preload meals may be provided as a standard meal with normal household ingredients (Stubbs *et al.*, 1996), or as a modified food (Vozzo *et al.*, 2003), often in liquid form (Bowen *et al.*, 2006c). A modified food produces a more accurate study meal because of inherent variations in the production and processing of standard food ingredients. Variability in meals due to differences in composition, such as palatability, fibre content, and energy density can be more precisely controlled when using modified foods. Visual appearance of study meals can be more comparable and differences in the composition of the meal

are often indistinguishable to subjects (Latner & Schwartz, 1999). Modified food preloads are frequently presented as a liquid which does not represent the typical form in which food is generally consumed, so results from these studies cannot easily be related to a normal eating environment. Providing a preload meal in liquid form may also alter measurements of glucose, insulin, satiety hormones and gastric emptying due to interactions between the meal and receptors in the gastrointestinal tract (Bowen *et al.*, 2006c).

The percentage of energy provided from each macronutrient differs between studies and may be very different from a standard diet. For high-protein meals, the percentage of energy from protein is often higher than is possible to achieve with standard meals, nor appropriate on a long-term basis. For example Latner and Schwartz (1999) compared a meal containing 71.5% of energy from protein to a meal comprised of 99% of energy from carbohydrate (Latner & Schwartz, 1999). The total energy provided at a preload meal also differs. Macronutrient influences on energy intake appear to be dependent on meal size; a change in energy intake is more likely if more than 1 MJ of the macronutrient is provided (Stubbs *et al.*, 1996; Poppitt *et al.*, 1998) suggesting a possible critical threshold required to influence satiety. A high-energy preload meal may saturate effects on satiety and blood parameters and reduce any effect between meals (Lang *et al.*, 1998; Lang *et al.*, 1999) while a low-energy preload may not alter satiety sufficiently to produce detectable effects on subsequent energy intake (Porrini *et al.*, 1997).

The timing and nature of the test meal may determine whether a preload influences subsequent energy intake. A longer period between the preload and the test meal may

more accurately reflect the effect of the preload on energy intake (Raben *et al.*, 2003), while providing the preload and the test meals at habitual eating times replicates usual meals and translates more reliably to habitual eating conditions (Stubbs *et al.*, 1996). Anderson et al demonstrated an increase in consumption of food at a test meal when the experiment was performed later in the morning (0830 versus 1100h) (Anderson *et al.*, 2004). The sensitive moment in time to offer a test meal has been determined by the latest time point after a preload meal where the measured parameters remain significantly different {Veldhorst, 2009 #972} {Veldhorst, 2009 #1063}.

Increasing the variety of foods presented at the test meal stimulates food intake (Rolls *et al.*, 1981; Stubbs *et al.*, 2001) and there is potential for this effect to exceed the satiating effect of the preload meal (Lang *et al.*, 1999).

Some studies do not provide a test meal but concentrate on measurements of satiety through visual analogue scales or post-prandial blood glucose and insulin levels after the preload meal (Nuttall & Gannon, 1990), but no information about energy intake. A further limitation of many preload studies is the lack of a control meal (Barkeling *et al.*, 1990; Poppitt *et al.*, 1998; Latner & Schwartz, 1999) that replicates the composition of subjects' habitual diet.

### 3.1.2 Studies of protein type in a preload design

In Chapter 1 studies with preload meals comparing protein, fat, and carbohydrate were described, and demonstrated that an increase in the proportion of protein in a meal increases satiety and, in some settings, leads to a subsequent reduction in energy intake.

Just as the impact of carbohydrate or fat may vary with the characteristics of the food, e.g. glycaemic index or fibre content of carbohydrate, or fatty acid composition of fat, so the amino acid profile of protein or other characteristics of different protein sources may have a differential impact on appetite, and protein type should therefore be considered in more detail. In mixed macronutrient preload studies, protein is usually meat-based or a mixture of proteins from different sources. Comparisons between types of protein are limited and inconclusive. Few studies have provided whole-food meals to measure satiety or energy intake with different sources of protein, with most opting for modified forms of protein meals or drinks. Preload studies investigating the effect of different protein type on energy intake and satiety are presented in Table 3.1.1.

Williamson *et al* (2006) compared pasta meals containing chicken, mycoprotein or tofu with 17% of energy provided by protein (Williamson *et al.*, 2006). The meals were controlled for energy density but fibre was higher in the mycoprotein meal than in the other two. Energy intake was significantly reduced after the mycoprotein and tofu meals (12.3% and 14.5% reduction respectively) compared to the chicken meal, with no compensation for this reduction at a subsequent meal four hours later. No difference was seen in the visual analogue ratings for hunger and fullness between these meals despite the difference in energy intake. Energy intake had previously been found to be 24% lower throughout the day after a meal containing mycoprotein, than a meal containing chicken, and 16.5% lower the following day, using self-reported weighed food diaries (Turnbull *et al.*, 1993). A reduction in visual analogue ratings for prospective food consumption and desire to eat was seen three hours after the study meal. In this study it was proposed that reductions in energy intake and increased satiety sensations after

mycoprotein consumption compared to chicken may have been due to the higher fibre content of mycoprotein, as fibre has been shown to enhance satiety in other preload studies (Hulshof *et al.*, 1993; Delargy *et al.*, 1997).

Modified forms of protein incorporated into soup or drinks have shown a reduction in energy intake at a test meal 90 minutes following whey compared to casein (Hall *et al.*, 2003). In contrast another study demonstrated no difference in energy intake after whey and casein liquid preloads (Bowen *et al.*, 2006c) possibly due to the smaller energy content of the meal (1 MJ) and a longer time to the preload meal (180 min). Lang *et al*, 1998, showed no difference between egg white, cow milk, pig gelatine, soy protein, pea protein, or wheat protein in energy intake at an *ad libitum* dinner eight hours after a test meal with a high-protein 5.2 MJ preload (Lang *et al.*, 1998), or when the energy content of the preload was reduced (3.6 MJ) (Lang *et al.*, 1999).

Subjective measures of satiety have produced conflicting results, perhaps reflecting their lower precision relative to objective measures of EI. When 50 g of beef, chicken or fish were provided to 6 lean men, satiety ratings were greater after fish than other protein sources (Uhe *et al.*, 1992). Uniquely, in this study subjects consumed only the protein source rather than a whole meal; therefore the appearance of the meals was very different. Longer satiety and less hunger were demonstrated after gelatine compared to casein in a 3.6 MJ meal (Lang *et al.*, 1999), but not in a 5.2 MJ meal (Lang *et al.*, 1998) perhaps due to a saturation effect of the higher energy meal. Hall *et al* (2003) measured energy intake and satiety in two studies with the same whey or casein preload and observed greater satiety after whey only in the second study, attributed to a difference in

study population (Hall *et al.*, 2003). Bowen *et al* (2006) demonstrated no differences in satiety scores between whey and casein after a 1 MJ liquid preload. However, this experiment was performed in overweight subjects who may have different responses to satiety scores irrespective of the preload provided (Bowen *et al.*, 2006a).

An increase in the post-prandial insulin area under the curve (AUC) has been seen after soy compared to gelatine (Lang *et al.*, 1999) and after cottage cheese compared to egg white, but the associated reduction in plasma glucose was not significant (Nuttall & Gannon, 1990). In contrast, no differences in glucose or insulin levels were demonstrated after protein loads of beef, chicken or fish (Uhe *et al.*, 1992) or when comparing whey and casein (Hall *et al.*, 2003, Bowen, 2006 #749)}. The effect of protein type on plasma glucose and insulin therefore remains to be determined.

Altering gastric emptying is one mechanism by which protein may influence appetite. Park *et al* (2007) assessed gastric emptying using scintigraphy after subjects consumed diets high in protein (energy from: 36%P, 40%C, 24%F), fat (energy from: 16%P, 40%C, 44%F), or carbohydrate (energy from: 16%P, 60%C, 24%F), compared to a standard diet (energy from: 20%P, 50%C, 30%F), over a two week period (Park *et al.*, 2007). No difference in the rate of gastric emptying of the solid meal was observed after a standard labelled-egg meal. In two studies whey and casein preloads were investigated using plasma paracetomol levels as a marker of the rate of gastric emptying. Hall *et al* (2003) demonstrated an initial rapid gastric emptying after a meal of casein, then a subsequent slower rate than after eating whey (Hall *et al.*, 2003) with lower energy intake and greater satiety measured after whey compared to casein, suggesting that the initial slower gastric

emptying after the whey meal reduced energy intake. Bowen *et al* (2006) found no difference in gastric emptying between whey and casein but gastric emptying was slower after these protein preloads than after glucose and lactose preloads (Bowen *et al.*, 2006c). A reduction in energy intake and satiety was also present after the protein preloads compared to the glucose preload implying that altering the rate of gastric emptying may be an important mechanism in the relationship between protein and satiety.

Additionally, protein type may influence appetite and energy intake through post-prandial secretion of gastrointestinal hormones. Ghrelin secretion is reported to increase after meat protein and decrease after consumption of dairy proteins (Karhunen *et al.*, 2008). In 9 lean subjects provided with casein or whey protein liquid preloads GLP-1 secretion was 65% higher after the whey protein than casein, and a corresponding greater satiety after whey was observed (Hall *et al.*, 2003) suggesting that gastrointestinal digestion and absorption of different types of protein is important for their subsequent satiety effect. Secretion of PYY and PP after meals of different protein type is not known, however as protein appears to stimulate PYY (Batterham *et al.*, 2006) and PP (Tomita *et al.*, 1989) release it is possible that protein type has a role in differential secretion of these hormones and the subsequent satiety effects.

**Table 3.1.1 Preload meals investigating protein type**

| | STUDY DESIGN | | | RESULTS | | |
|---|---|---|---|---|---|---|
| **Reference** | **Subjects** | **Intervention** | **Preload meal** | **Hunger/Satiety Results** | **% Change in Energy Intake** | **Other Results** |
| Lang *et al*, 1998 | n = 12<br>lean males<br>crossover | Standard breakfast<br>protein lunch (modified to control palatability, fibre, macronutrients): – onion soup and chocolate desert<br>*ad lib* dinner 8 h later | Egg-white protein<br>Cow-milk casein,<br>pig gelatin<br>soy protein, pea protein<br>wheat protein, no control<br>5.2MJ meals, 70g protein<br>P 22.4%<br>C 61.2%<br>F 16.4% | No difference in hunger and satiety ratings | No difference in EI at dinner or the day after,<br>In 24 h post meal P intake lower, C intake higher | glucose curve – no difference<br>insulin curve – no diff |
| Lang *et al*, 1999 | n = 9<br>lean males<br>crossover | Standard breakfast<br>protein lunch (modified to control palatability, fibre, macronutrients) – onion soup and chocolate desert<br>*ad lib* dinner 8 h later | cow milk casein,<br>pig gelatin<br>soy protein<br>3.6 MJ 50g P<br>1.8MJ 25g P<br>P 22.4%<br>C 61.2%<br>F16.4% | Longer satiety and less hunger after gelatine compared to casein in 3.6MJ meal | No difference in EI at dinner or the day after,<br>In 24 h post meal P intake higher especially after 1.8MJ meal | Investigated saturation effect of Lang 1998<br>Glucose curve - no difference (non-significant reduction in glucose after soy)<br>Insulin curve – increased AUC after soy compared to gelatine, later insulin peak with gelatine and casein |

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| | | | | | | Glucagon – no difference |
| Uhe *et al*, 1992 | n = 6<br>lean males<br>crossover | protein source only (not a meal)<br>no test meal | 50 g protein<br>beef<br>chicken<br>fish | greater satiety with fish | | Amino acids slower rise to peak after fish<br>No difference in insulin/glucose<br>Fish – slower decrease in tryptophan:LNAA ratio (? role of serotonin in satiety) |
| Nuttall and Gannon, 1990 | n = 7<br>lean males | Preload at 0800 h, blood samples up to 8 h after | Water – control<br>50 g cottage cheese<br>50 g egg white | | | Glucose lower after cottage cheese than egg white (ns)<br>Insulin peaked and remained elevated for 5 h after cottage cheese and increased AUC<br>a-amino-nitrogen and urea nitrogen greater peak after cottage cheese<br>c-peptide peaked but fell rapidly to control after cottage cheese<br>glycogen similar<br>NEFA transient decrease after egg and cheese correlated with |

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| | | | | | | insulin<br>Triglycerides peaked after 2 hrs with cottage cheese |
| Anderson *et al*, 2004 | Lean males<br>Ex 1 n=13<br>Ex 2 n=22<br>Ex 3 n=10<br>Ex 4 n=13 | Expt 1: water, sucrose, egg albumen, whey, soy protein. EI at 1 h<br>Expt 2: water, 50 g whey, egg albumen. EI at 1h<br>Expt 3: Water, 50 g whey. EI at 2 h<br>Expt 4: control, soy, soy with high or low GI amylose or glucose. EI at 1 hr | 833 kJ drinks | | Ex 1 soy EI 14% lower than control (water)<br>Whey EI 23% lower<br>Egg no diff to water<br>Ex 2 similar<br>Ex 4 soy EI 22% lower than control, 12% lower than soy and glucose | |
| Hall *et al*, 2003 | Study 1 n=16 lean male and female<br>Study 2 n=9 lean crosover | Study 1 Liquid preload followed by ad libitum buffet meal at 90 min<br>Study 2 liquid preloads contained paracetomol followed by fixed meal at 90 min | 1.7 MJ<br>48g protein<br>powdered whey or casein made into liquid preloads | Hunger and satiety VAS – no difference study 1<br>Fullness increased after whey in study 2 | EI 19% lower following whey than casein | Study 2 gastric emptying - faster initially after casein then slower than whey<br>Insulin / Glucose – no diff<br>GLP-1/ GIP/ CCK/ AA – higher after whey |
| Bowen *et al*, 2006 | n = 19<br>overweight males<br>mean BMI 32.1 kg/m$^2$ | Liquid preload 0900 then 1500mg paracetomol<br>Artificially sweetened chocolate drink controlled for energy, energy density, palatability, and consistency. | 1 MJ meals<br>whey protein 55 g<br>calcium caseinate 55 g<br>P 83%<br>C 15% | Appetite higher after glucose | No difference between whey and casein.<br>EI higher after glucose<br>No difference between lactose or protein. | Gastric emptying – slower after protein<br>CCK levels higher after protein<br>Ghrelin returned to baseline |

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|---|---|---|---|---|---|---|
| | crossover | Test meal at 180 min | F 2%<br>glucose 60 g GI = 100<br>lactose 56 g GI = 43<br>P 12%<br>C 87%<br>F 1% | | | faster after glucose than after protein and lactose.<br>Glucose higher after glucose<br>Insulin no difference, followed ghrelin curves until 120min when insulin returned to baseline but ghrelin did not.<br>Amino acids differed between whey and casein but no corresponding difference in EI. |
| Williamson *et al*, 2006 | n = 42<br>overweight females<br>mean BMI 27.4 kg/m$^2$<br>crossover | Standard breakfast: 2.01MJ, 10% protein, 60% carbohydrate, 30% fat, 3.3 g of fibre.<br>Preload 4 h after breakfast eaten within 20 min, pasta meal, unmodified food<br>Test meal (Ham sandwiches) after 20 min<br>Test dinner 4.5h after lunch (MSSP meal)<br>Controlled for energy density and palatability<br>Fibre higher in mycoprotein meal | Mycoprotein<br>Tofu<br>Chicken<br>220g meals<br>1.9 MJ<br>P 17%<br>C 65%<br>F 17% | VAS no difference in hunger and fullness scores | EI 12.3% lower after mycoprotein and 14.6% lower after tofu than chicken preload | Universal Eating monitor<br>Macronutrient self-selection paradigm (MSSP) no compensation at dinner after reduced intake at lunch. Less high fat, high sugar foods consumed after tofu. |

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|---|---|---|---|---|---|---|
| Turnbull *et al*, 1993 | n = 13<br>lean females<br>crossover | Fasted until lunch study meal.<br>Whole-food meal<br>Weighed diary records of food consumption on the study day and the next day<br><br>Fibre higher in mycoprotein meal | Mycoprotein<br>Chicken<br>44g P<br>2.4 MJ<br>P 30%<br>C 55%<br>F 15% | VAS<br>Significant reduction in prospective food consumption and desire to eat 3 h after mycoprotein | EI 24% lower after mycoprotein on the study day, 16.5% lower the next day | |

Abbreviations: P, Protein; C Carbohydrate; F, Fat; HP, High protein; HC, High carbohydrate; HF, High fat; EE, Energy expenditure; EI, Energy intake; AUC, Area under the curve; NEFA, Non-esterified fatty acids; AA, Amino acids; CCK, Cholecystokinin; GIP, Glucose-dependent insulinotropic polypeptide; GLP-1, Glucagon-like peptide 1; GLP-2, Glucagon-like peptide 2; VAS, Visual analogue scale; NS, Not significant; RMR Resting metabolic rate.

### 3.1.3 Influence of protein type on energy balance and metabolic risk

In addition to putative effects on energy intake, there is emerging evidence that specific types of protein may exert differential effects on glucose control, insulin resistance and cardiovascular risk factors.

#### *3.1.3.1 Nuts*

Nuts provide energy, protein, fibre, essential fatty acids and certain vitamins and minerals, and are an important diet component in many different cultures. Many are, however, energy dense and high in fat. As evidence has emerged about the cardiovascular benefits of eating nuts there has been concern that weight gain may be a detrimental side effect. In fact, the data to date suggest a beneficial effect on body mass of people eating nuts. Epidemiological data indicate that frequent nut consumption is associated with no change or reduction in body mass (Fraser *et al.*, 1992; Hu *et al.*, 1998; Sabate, 2003). Intervention studies have investigated the effect on body mass by providing a supplement of nuts without other dietary advice. Over six months, a 1340 kJ daily supplement of almonds produced no change in body mass but a substantial change in fatty acid intake (which may contribute to the cardiovascular benefits of nuts) (Fraser *et al.*, 2002a) (Fraser *et al.*, 2002b). In a 12-month crossover study a 6-month, 965 kJ daily walnut supplement produced a much lower than expected weight gain for the higher energy intake. Hollis and Mattes (2007) (Hollis & Mattes, 2007) provided a 1440 kJ almond supplement to twenty overweight women for 10 weeks. No change in body mass or body composition was reported and energy intake did not increase, suggesting a satiating effect during the almond supplementation. The subsequent dietary compensation accounted for 74% of the energy contributed by the almonds. Resting metabolic rate and total energy expenditure, measured by doubly labelled water, did not differ in subjects eating more

almonds. There was, however, a significant decrease in the digestibility coefficient of the diet, measured by faecal energy content, which accounted for a further 7% of the energy contributed by the almonds.

During weight loss with a low-calorie liquid diet supplemented with almonds or complex carbohydrates in 65 subjects Wien *et al* (2003) demonstrated a greater, more sustained weight loss in the almond-eating group over 24 weeks with a significantly lower waist circumference and fat mass than the carbohydrate group despite similar energy consumption in the two groups (Wien *et al.*, 2003).

Diets rich in nuts have additionally been associated with a lower risk of cardiovascular disease. There is now strong epidemiological evidence from the Adventist Health Study (Fraser *et al.*, 1992), the Nurses' Health Study (Hu *et al.*, 1998), the Physicians' Health Study (Albert *et al.*, 2002), and the Iowa Women's Health Study (Ellsworth *et al.*, 2001) that frequent consumption of nuts lowers the risk of fatal and non-fatal coronary heart disease. Several intervention studies have shown that nuts improve lipid profiles with normal and hypercholesterolemia (Sabate *et al.*, 1993), (Rajaram *et al.*, 2001), (Lovejoy *et al.*, 2002), (Sabate *et al.*, 2003), (Garg *et al.*, 2003), (Sheridan *et al.*, 2007). Proposed mechanisms for the beneficial effect of nuts on cardiovascular disease have been related to an increase in unsaturated fatty acid consumption, increased fibre, reductions in glycaemic index, increased vitamin E and magnesium and the role of arginine in increasing nitric oxide-stimulated vasodilatation (Rajaram & Sabate, 2007) (Hu *et al.*, 1998; Jiang *et al.*, 2002).

When investigating the risk of developing type 2 diabetes, nut consumption may also be beneficial. A prospective cohort study of 83,818 women in the Nurses' Health Study, followed for 16 years, found an inverse association with frequency of nut consumption and risk for developing type 2 diabetes, with a 30 % lower risk in those who consumed nuts five or more times a week (Jiang *et al.*, 2002). In established type 2 diabetes there is some evidence that nut supplementation improves glucose metabolism, with a greater reduction in fasting insulin and requirement for diabetes medication (Wien *et al.*, 2003). Lower post-prandial glucose and insulin curves and reduced post-prandial oxidative damage have been seen following a nut preload, compared to a meal of rice or potatoes with added butter and cheese to match macronutrient composition (Jenkins *et al.*, 2006).

### *3.1.3.2 Dairy products*

The effect of dairy products on body mass remains controversial as dairy products may be high in fat, particularly saturated fat, which can be detrimental to weight regulation and cardiovascular disease risk, but have, in some observational studies, contributed to loss of body mass in the overweight and obese (Zemel, 2005). Epidemiological studies describe inconsistent relationships between intake of dairy products and weight. No effect of dairy or calcium intake on body weight or weight change was seen over 12 years in the Health Professionals Study despite those in the highest quintile of calcium intake being more physically active (Rajpathak *et al.*, 2006). Moreover, men with the largest increase in high-fat dairy products over the study period gained significantly more weight than men who decreased their intake. In a similar-sized longitudinal study of 19,352 Swedish women BMI was higher in subjects with a low intake of whole milk and sour milk, cheese and butter, but lower in subjects with a low intake of low-fat milk and sour milk

(Rosell *et al.*, 2006). Women who maintained a higher intake of dairy products had a lower risk of gaining weight over the mean 8.8 y follow-up than women who decreased their intake. Conversely an association between cheese intake and a higher BMI was seen in 2064 individuals in the Hoorn Study (Snijder *et al.*, 2007). In a comparison of two high-protein weight loss diets, one high in dairy protein and the other a mixed protein diet, there was no difference in weight loss between the two groups, or any difference in other metabolic parameters (Bowen *et al.*, 2005). A recent opinion by the European Food Safety Authority (EFSA) concluded that a cause and effect relationship is not established between the daily consumption of dairy foods (milk, cheese and yoghurt) and a healthy body weight in children and adolescents, due to a lack of intervention studies to examine a causal relationship. Additionally there is insufficient data to establish a specific level or frequency of consumption of dairy foods associated with any specific effect on body weight (EFSA, 2008).

The putative benefit of dairy products is thought to be increased calcium intake, with possible additional benefits of milk proteins. Increased calcium intake down-regulates parathyroid hormone and 25-dihydroxyvitamin D [1,25-(OH2)D] and therefore reduces intracellular calcium. Lower intracellular calcium stimulates lipolysis and inhibits lipogenesis in adipocytes, thereby reducing fat mass (Zemel, 2002). In addition, decreased intracellular calcium concentrations may reduce blood pressure by lowering vascular smooth muscle tone and peripheral vascular resistance. Some milk proteins inhibit angiotensin-converting enzyme (ACE) activity, which may explain the antihypertensive effect, and may contribute to regulation of adipocyte lipogenesis (Shahar *et al.*, 2007).

The potential effect of dairy calcium on body mass has been investigated in intervention studies, although often in the context of energy restriction. Adding two portions of yoghurt to the diet of obese African-Americans over 1 y resulted in a reduction in body weight of 4.9 kg. Subsequently 32 obese adults were randomised to a 2000 kJ/d deficit weight loss diet for 6 months with standard dietary advice, a high-calcium diet, or a high-dairy diet. Those in the high-dairy group reduced body mass, fat mass and waist circumference significantly more than the high-calcium group, who reduced these parameters more than the standard diet group (Zemel *et al.*, 2004). Therefore, the beneficial effects appear greatest when dairy products are consumed in entirety, although calcium supplementation may also influence body mass loss.

An effect of high calcium on body mass has not been consistently observed. In a comparison of two high-protein weight loss diets, one high in dairy protein and the other a mixed protein diet, there was no difference in weight loss between the two groups, or any difference in other metabolic parameters (Bowen *et al.*, 2005). Similarly, in 54 obese men assigned an energy restricted diet high (1200mg/d) or low (500 mg/d) in dairy calcium, no difference in weight loss was demonstrated between the diets (Harvey-Berino *et al.*, 2005). When the calcium was supplied in tablet form, a 1000 mg/d calcium supplementation compared with placebo in 100 women did not produce a difference in change in body mass or fat mass (Shapses *et al.*, 2004). Recently, additional dairy calcium enhanced weight loss in 259 overweight people with type 2 diabetes (Shahar *et al.*, 2007). A possible explanation for weight loss is due to increased fat loss in faeces. A short-term increase in dairy calcium did not alter 24-h energy expenditure but increased faecal fat and energy elimination by 350 kJ/day (Jacobsen *et al.*, 2005).

A beneficial effect of eating dairy products on features of the metabolic syndrome and cardiovascular disease is less controversial. The 10-year longitudinal Coronary Artery Risk Development in Young Adults (CARDIA) study showed an inverse association between dairy intake and development of features of the metabolic syndrome in overweight subjects, with a reduction in development of obesity, abnormal glucose homeostasis, elevated blood pressure and dyslipidaemia (Pereira *et al.*, 2002). An association between a high consumption of low-fat dairy products and a lower risk for developing type 2 diabetes was also seen in the Health Professionals Study (each serving-per-day increase in total dairy intake was associated with a 9% lower risk for type 2 diabetes) independent of body weight (Choi *et al.*, 2005). A 24-week high-dairy weight loss diet improved glucose tolerance in overweight subjects compared to high-calcium or standard diets and modestly improved systolic blood pressure (Zemel *et al.*, 2004). An association between dairy and a decrease in blood pressure (Snijder *et al.*, 2007) and the diagnosis of the metabolic syndrome (Lutsey *et al.*, 2008) has also been reported.

#### *3.1.3.3 Soy*

Most research investigating the effect of protein type on body mass and metabolic parameters has focused on animal rather than vegetable sources of protein such as soy. Studies of diets rich in soy protein have predominantly investigated serum lipids and other markers of metabolic risk, rather than a specific effect on weight regulation. A soy based energy-restricted diet in 30 obese adults who consumed either soy protein as the only protein source or a traditional diet of animal and plant protein for 8 weeks produced a similar degree of weight loss (Liao *et al.*, 2007) and significantly reduced LDL cholesterol concentration, but not waist circumference or blood pressure. Similarly, 47

overweight women, provided with either dietary counselling alone or with soy protein products as their main source of protein in addition to dietary counselling, over 12 weeks, showed no difference in weight loss, change in fat mass, waist circumference, lipid, glucose or insulin concentrations (St-Onge *et al.*, 2007). Soy milk and skimmed cows' milk added to an energy restricted diet, in a small study of 14 overweight women, produced the same degree of weight loss and loss of fat mass (Lukaszuk *et al.*, 2007). In a study designed to investigate the hormonal effects of soy food and a low-fat diet, 57 women were randomised to a very low-fat diet, a control diet or a control diet supplemented with soy-based foods. Subjects were not counselled to lose weight but small, equal amounts of weight were lost in each group over 8 weeks (Wu *et al.*, 2005). In one of the only studies to show an effect of soy products on weight loss Diebert *et al*, 2004, randomised 90 overweight subjects to receive lifestyle education, or a high-soy-protein diet with or without a physical activity programme for 6 months (Deibert *et al.*, 2004). Subjects in the high-soy-protein groups lost significantly more weight and had a greater loss of fat mass than the lifestyle education group, possibly due in part to the soy group being provided with a more prescriptive energy-restricted diet (and soy-based meal replacements) than those provided counselling alone.

The beneficial effect of soy on cholesterol is clearer. A meta-analysis in 1995 concluded that soy protein consumption reduced total and LDL-cholesterol by 9% and 13% respectively (Anderson *et al.*, 1995). 34 of the 38 studies analysed showed a reduction in cholesterol and, in those studies that did not, subjects had low baseline cholesterol. These findings formed the basis of a health claim approved by the Food and Drug Administration (FDA) that 'soy protein included in a diet low in saturated fat and

cholesterol may reduce the risk of CHD by lowering blood cholesterol levels' (http://vm.cfsan.fda.gov/~lrd/fr991026.html). This claim recommends consumption of 25 g soy protein per day, as part of a low-saturated-fat, low-cholesterol diet, to help reduce risk of coronary heart disease. The mechanism for reduction in cholesterol levels is unclear but may be due to the oestrogenic effects of soy proteins which contain isoflavones and phytoestrogens (Anderson *et al.*, 1995). A further meta-analysis investigated the effect of soy protein containing isoflavones (Zhan & Ho, 2005). After analysis of 23 trials, soy protein was associated with significant decreases in serum total cholesterol (by 3.77%), LDL cholesterol (by 5.25%), and triacylglycerols (by 7.27%) and significant increases in serum HDL cholesterol (by 3.03%). The change in cholesterol was dependent on the baseline cholesterol concentration. Reduced cardiovascular disease with an increase in soy intake is primarily attributable to an improvement in lipid profiles, however other possible mechanisms of a soy diet are reducing blood pressure and lowering glucose concentrations.

Yang *et al* (2005) investigated the effect of soy on blood pressure in a large longitudinal study. Women aged 40 – 70 y who consumed more than the 25g per day FDA recommendation had a significantly lower systolic and diastolic blood pressure than women who consumed less (Yang *et al.*, 2005). They proposed that soy isoflavones reduce oxidative stress and inflammation and may stimulate the production of nitric oxide to enhance vasodilatation and hence reduce blood pressure.

Using the method to determine glycaemic index (FAO/WHO, 1998) 6 commercial products rich in soy (2 chocolate soy bars, soy spaghetti, 2 chocolate soy shakes and soy

protein chips) were tested against the glucose reference in 10 subjects to investigate the glucose and insulin response to soy food (Blair *et al.*, 2006). Subjects had significantly lower glucose and insulin excursions after consuming the test foods in comparison to glucose. Aall but one of the 6 foods tested (the soy protein chips) had a glycaemic index below 55. In a comparison of cod, cows' milk and soy protein, von Post-Skagegard (2006), demonstrated a significantly lower area under the glucose curve in subjects consuming a soy protein meal than after cod protein, and a similar glucose response to milk. Insulin levels were lower after the soy than milk diets but higher than after cod (von Post-Skagegard *et al.*, 2006).

### 3.1.4 Summary

Evidence from preload studies suggests that protein may be more satiating than other macronutrients but less is known about the relative effects of different types of protein. Certain types of protein are associated with improvements in metabolic risk factors and possibly regulation of body mass. Possible mechanisms include differences in post-prandial metabolism, which may modulate both metabolic risk and, through feedback loops to appetite, body mass control. This study investigates, in a preload design, whether incorporating different protein types into a moderately high-protein meal differentially alters appetite and subsequent energy intake.

### 3.1.5 Objective

The primary objective of this study was to determine whether isoenergetic high-protein preloads (33% of energy from protein), based on meat, dairy, nuts and legumes, or soy or

a preload of standard composition (15% of energy from protein), exert differential effects on satiety and subsequent energy intake.

The secondary objective was to make a preliminary investigation of the post-prandial metabolic (glucose, insulin and fatty acids), and gastric (gastric emptying) response to different protein sources and in comparison to a control treatment, as potentially important variables to explain the differences in appetite control and weight regulation associated with different foods.

## 3.2 Methods

This experiment was performed at MRC Human Nutrition Research (HNR) between July 2005 and May 2006. The study was approved by Peterborough & Fenland Local Research Ethics Committee in June 2005 (Ref: 05/Q0106/37).

### 3.2.1 Subjects

#### *3.2.1.1 Sample size*

Prior estimates of effect size and the likely standard deviation were limited by a scarcity of studies using a crossover design to test various preload meals with an isoenergetic control. Accordingly, the sample size calculation was based on Warren *et al* (2003) in which 37 subjects were given low or high-glycaemic index breakfasts, or a control breakfast, in a cross-over trial (Warren *et al.*, 2003). The effect size was based on a mean

difference in energy intake of 10%. To detect an effect of this magnitude at a significance level of 0.05 and power of 80%, a sample size of 30 subjects was required.

*3.2.1.2 Recruitment*

Subjects were recruited from the general population in a number of different ways. A search was performed on the HNR volunteer database. This database contains basic anthropometric and medical information about volunteers interested in research to allow selection of potentially suitable subjects. A letter, approved by the ethics committee (Appendix I.2.4), was sent to all lean healthy subjects inviting them to participate in the study. An advertisement (Appendix I.2.6) was placed on a graduate university student email newsletter. Advertisements were also placed in local libraries, doctors' surgeries and supermarkets. Information sheets were distributed through unit-wide information stands at Cambridge Science week, at local shopping malls, and at HNR volunteer open evenings. Information sheets and advertisements were also placed in the volunteer suite lounge for subjects who were participating in other research in the unit.

Potential subjects contacted the chief investigator to discuss the study in more detail. Those wishing to participate completed a brief telephone-screening questionnaire (see Appendix I.2.3) to determine eligibility. Subjects were then invited to HNR. Written informed consent was obtained. A brief questionnaire enquired about dieting history, guilt associated with food, and not eating when hungry, to exclude restrained eaters. Body mass, height and blood pressure were measured according to the methods described in Chapter 2. Body mass index was calculated to ensure eligibility.

The general practitioners of all eligible subjects wishing to participate in the study were informed (see Appendix I.2.5). They were asked to contact the chief investigator if there was any medical reason that would prevent participation in the study. All clinically relevant results were reported back to the GP with the consent of the subject.

*3.2.1.3 Eligibility*

Eligible subjects were aged between 18 and 70 years. They were lean (BMI 18-25 $kg/m^2$), non-smokers and took no medications or had any medical conditions that excluded them from participating. They were willing to eat all the study meals and did not report any unusual or restrictive eating habits.

Subjects were excluded if they had acute or chronic medical conditions or were taking medications that would interfere with satiety, gastrointestinal function, or glucose metabolism. The oral contraceptive pill and hormone replacement therapy were allowed if continuous use had been for three months or longer. Subjects were also excluded if they were pregnant, lactating or planning to conceive, and those unable to eat all of the study foods (because of dietary preference, food allergy or intolerance).

Subjects were asked not to alter lifestyle factors for the duration of the study visits, to continue their usual diet and exercise regime and to not start or stop taking any dietary supplements four weeks prior to participation in the study or for the duration of the study. Pre-menopausal women were studied during the same phase of their menstrual cycle as changes in energy balance occur during the cycle, particularly an increase in energy

intake and body mass in the pre-menstrual (late luteal) phase (Buffenstein *et al.*, 1995) (Davidsen *et al.*, 2007).

### 3.2.2 Study Design

The study was a five period randomised crossover of four preload meals high in protein and a control preload of standard protein. The predominant protein source in the high-protein preloads was from meat, dairy, nuts and legumes, and soy. The preload meal was labelled with [1-$^{13}$C]-Octanoic acid in order to measure gastric emptying. Subjects visited HNR to consume each of the five preload meals. At baseline and after the preload meal measurements were taken of satiety, gastric emptying and metabolic responses to the preload. An *ad libitum* test meal was served three hours after the preload and the energy of the food consumed was calculated.

Subjects attended HNR on five separate occasions in random order, with at least one week between visits, on each occasion after a 12 h fast. Random allocation to each visit was performed using random number tables. Subjects had maintained their usual diet and exercise regime and had not consumed alcohol for 24 h prior to their visit. Study visits were scheduled away from celebrations and social engagements to prevent the study day being affected by unaccustomed eating or sleeping the previous day. Transport to HNR was by car or taxi to avoid vigorous exercise on the subject's investigation days.

Participants were advised that they were participating in a study to investigate the effect of different meal types on the metabolic response to eating. The covert nature of the study outcome was designed to ensure that subjects did not change their eating behaviour

because of knowing what was being studied and was conducted according to British Sociological Association 2002 guidelines on the use of covert research in such circumstances (http://www.socresonline.org.uk/info/ethguide.html).

The protocol for each investigation day is presented in Table 3.2.1. On arrival at HNR subjects were interviewed to ensure that were fasted, had not had any recent illnesses or taken any medication to exclude them from the study. They recalled their diet from the previous 24 h and indicated whether it diverted from their habitual diet. Body mass, height and blood pressure were recorded at the initial visit and body mass was measured at subsequent visits according to the method in Chapter 2. An intravenous cannula was inserted (see Chapter 2 for details). A breath test was taken 12 minutes prior to the preload meal and just before the preload meal was served. Three baseline blood samples were taken through the intravenous cannula 10, 5 and 1 minute before the preload meal. Visual analogue scales for appetite were completed 10 and 1 minute before the preload. The preload meal was served in private cubicles in the volunteer suite lounge. Subjects were asked to consume the entire meal within 15 minutes. They were allowed water to drink during the preload and throughout the study day.

**Table 3.2.1 Summary of experimental design of investigation days**

| Time (min) | | Blood Sample | Breath Test | Visual Analogue Scale |
|---|---|---|---|---|
| -30 | Weight measured<br>Questionnaire completed<br>IV cannula inserted | | | |
| -12 | | | X | |
| -10 | | X | | X |
| -5 | | X | | |
| -1 | | X | X | X |
| 0 | Preload Meal | | | |
| 12 | | | X | |
| 24 | | | X | |
| 30 | | X | | X |
| 36 | | | X | |
| 48 | | | X | |
| 60 | | X | X | X |
| 72 | | | X | |
| 84 | | | X | |
| 90 | | X | | X |
| 96 | | | X | |
| 108 | | | X | |
| 120 | | X | X | X |
| 132 | | | X | |
| 144 | | | X | |
| 150 | | X | | X |
| 156 | | | X | |
| 160 | | | | |
| 168 | | | | |
| 180 | | X | X | X |
| | Test Meal | | | |
| 192 | | | X | |
| 204 | | | X | |
| 210 | | | | X |
| 216 | | | X | |
| 228 | | | X | |
| 240 | | | X | X |
| 252 | | | X | |
| 264 | | | X | |
| 276 | | | X | |
| 288 | | | X | |
| 300 | | | X | X |
| 312 | | | X | |
| 324 | | | X | |
| 336 | | | X | |
| 348 | | | X | |
| 360 | | | X | |

Breath samples continued after the preload at 12-minute intervals for six hours. Subjects were given a timer set to alarm every 12 minutes and a rack of labelled tubes to blow into. Visual analogue scales were taken at 30-minute intervals for 5 hours. Blood samples were taken every 30 minutes until the last sample at 180 minutes. At this time the cannula was removed and the *ad libitum* test lunch was served. The lunch was eaten in private, without interruption, and subjects were allowed 30 minutes for their meal. Subjects were asked to eat until they felt comfortably full. A palatability questionnaire was completed after the preload meal and the test meal.

### *3.2.2.1 Meals*

Subjects consumed all preloads in random order on their five study visits. The composition of the five preload meals, one of standard protein and four of high-protein was calculated using UK food databases (McCance and Widdowson tables) (FSA, 2002) as described in Chapter 2. A summary of the meals is presented in Table 3.2.2 and details are shown in Appendix I.1.

All preloads provided 2.5 MJ metabolisable energy. The standard protein preload was the control and contained 15% of energy from protein, 34% from fat, and 51% from carbohydrate. Protein sources were meat and vegetables designed to resemble usual protein consumption. The four high-protein preload meals were composed of 33% protein, 34% fat and 33% carbohydrate. Fat was held constant to minimize the impact of manipulations on energy density. However it was not possible to ensure all relevant dietary factors were standardised given the inherent differences in different sources of protein, for example with respect to fibre. In order to test the effect of the different

protein types, the preloads were designed so that the predominant protein was the type being tested. The four protein types were meat, dairy, soy and a nuts and legumes mix. During recipe testing it became apparent that it was not possible to construct a suitable matched diet based on nuts alone because of the high percentage of fat. Adding lentils to the recipe provided more protein but disproportionately increased the carbohydrate level. In order to produce a nuts and legumes preload of the same composition as the other three, egg whites were added to the recipe. To provide consistency, they were then added to all preloads and the recipes were adjusted to equalise the macronutrient composition.

The preload meals were similar in appearance and presentation, each containing a protein and vegetable bake with potato topping. Ingredients other than the primary protein source were the same in each recipe with variation in specific quantities. Olive oil was used where additional fat was required in order to keep the acylated fatty acid content as even as possible, given the high amount of monounsaturated fat in the nuts and legumes preload. All ingredients were standard food items to ensure preloads resembled ordinary dishes. As far as possible ingredients were purchased in bulk for the entire study to limit variation in the food items used. The meals were prepared in the HNR volunteer suite kitchen following basic food hygiene standards. Meals were frozen, then defrosted and heated in the oven prior to each investigation day according to a standard protocol.

One egg yolk was incorporated into all preloads as a vehicle for the $^{13}C$ octanoic acid label to measure gastric emptying (see 3.2.3). The yolk was blended with the mashed potato topping so that an even amount of isotope would be eaten with each mouthful of food and measurement of gastric emptying would be consistent from the start of the meal.

Subjects were asked to eat the entire preload meal within 15 min at the start of the experiment day. Many subjects commented that the preload meal was a large amount of food to eat in the morning and were not able to complete the preload in the requested time. Some were not able to complete the preload after 30 min at which time the plates and serving dishes were removed and the remaining food was weighed.

An *ad libitum* test meal was provided at 180 min after the preload meal. Subjects were offered a large plate of pizza (9.4 MJ) and a large bowl of yoghurt (3.8 MJ) from which they could serve their own meal. Both dishes were comprised of 15% protein, 34% fat, and 51% carbohydrate. The pizza had an homogenous cheese and tomato topping and was cut into 32 small even-sized slices. This serving arrangement reduced the likelihood of subjects knowing what proportion of the pizza they had consumed. Minimizing interaction between cognitive cues and innate appetite control mechanisms was important as in a trial of the study design, one subject ate exactly half of one pizza while another ate a whole pizza, suggesting that eating patterns were strongly influenced by such habits, and would potentially be replicated in subsequent visits without following satiety cues. The yoghurt was full-fat strawberry yoghurt containing pureed fruit. The dishes were covertly weighed before and after the meal so that energy consumed could be calculated.

**Table 3.2.2 Summary of the composition of the preload meals**

| | | | | | | Fatty acids | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Protein g (% of total energy)** | **Fat g (% of total energy)** | **Carbohydrate g (% of total energy)** | **Energy kJ (% of total energy)** | **Dietary Fibre g** | **Saturated g** | **Monounsaturated g** | **Polyunsaturated g** | **Trans g** |
| **Meat Control** | 22.5 (15.3) | 22.9 (33.8) | 75.1 (50.9) | 2508 (100) | 8.3 | 4.5 | 13.6 | 2.8 | 0 |
| **Meat, high-protein** | 48.6 (33.0) | 23.4 (34.5) | 47.8 (32.5) | 2504 (100) | 5.0 | 5.8 | 12.6 | 2.3 | 0.2 |
| **Dairy** | 48.7 (33.1) | 23.1 (34.2) | 48.1 (32.7) | 2500 (100) | 3.7 | 7.8 | 11.1 | 2.0 | 0 |
| **Nuts and legumes** | 47.8 (32.1) | 23.8 (34.8) | 49.2 (33.1) | 2528 (100) | 12.0 | 3.1 | 12.5 | 5.7 | 0 |
| **Soy** | 48.6 (32.9) | 22.9 (33.8) | 49.0 (33.2) | 2506 (100) | 7.1 | 4.0 | 12.6 | 3.5 | 0 |

### 3.2.3 Measurement of gastric emptying

Gastric emptying was assessed by measuring enrichment of $^{13}C$ in breath $CO_2$. Isotopically labelled $^{13}C$ is incorporated into the medium-chain fatty acid octanoate and consumed as part of a study meal. $^{13}C$-octanoic acid remains bound to the solid phase of a meal, but after passing through the pylorus and into the small intestine is rapidly absorbed, released into the portal vein, transported to the liver where it is metabolised (Jackson & Bluck, 2005). Approximately half of the label is oxidized to $^{13}CO_2$, which is released in the breath after passing through bicarbonate pools and can be measured by continuous flow isotope ratio mass spectrometry.

In this study 100 µl [1-$^{13}C$]-Octanoic acid was added to a beaten egg yolk, which was cooked until solid in a dry frying pan to ensure binding of the isotope to the yolk lipids. The yolk was then blended to a fine consistency so that it could be added to the preload and consumed at an even rate throughout the meal. To collect the breath samples subjects were asked to gently blow through a drinking straw into 2 x 10 ml tubes for a count of 10 s or until water vapour appeared on the side of the tube. Breath samples were stored at room temperature until analysis by isotope ratio mass spectrometer.

### 3.2.4 Outcome measures

#### *3.2.4.1 Energy Intake*

Energy intake at the test meal was calculated from the weight of the food served minus that remaining.

*3.2.4.2 Hunger and Satiety scores*

Visual analogue scales to assess hunger, feeling of fullness, nausea, and mood were completed immediately prior to the preload meal (-10 and –1 min) and at 30, 60, 90, 120, 150 and 180 min, and 30, 60 and 120 min after the test meal. Participants were asked to place a vertical mark on a continuous 10 cm line to indicate their response to each question. (See Chapter 2 for detail and Appendix I.2.7 for the questionnaire).

*3.2.4.3 Palatability*

Visual analogue scales to assess palatability of the meal were completed after the preload meal and the test meal (Appendix I.2.8).

*3.2.4.4 Blood markers of metabolic risk and satiety*

Three 10 ml baseline blood samples were taken at –10, -5 and –1 min and a further six 10 ml post preload blood samples were taken at 30, 60, 90, 120, 150 and 180 min as indicated in Table 3.2.1. The samples were analysed for glucose, insulin, and non-esterified fatty acids.

*3.2.4.5 Gastric Emptying*

Two 10 ml breath samples were taken 12 min and 1 min prior to the preload meal and at 12-min intervals for the six-hour duration of the study.

*3.2.4.6 Data analysis*

The primary outcome measure of energy intake at the test meal was compared between preload meal types and analysed relative to the control preload using a mixed effects linear regression model for a crossover study that accounts for unbalanced randomisation and missing data.

Hunger and satiety ratings and palatability scores were plotted over time and were analysed for AUC to 300 min, AUC to 180 min (the test meal), peak score and time to peak score, minimum score and time to minimum score, and score at all timepoints. A mixed effect linear regression model was used to compare baseline and peak ratings and scores at 180 min. Area under the curve was plotted using two methods as discussed in Chapter 2 (model a included all area under the curve, model b subtracted any area below baseline from the area above the curve) and differences between groups was analysed using a mixed effect linear regression model.

Glucose, insulin, fatty acid data, were analysed for change from baseline and area under the curve using the same method for VAS scores.

$^{13}C$ enrichment values in the breath $CO_2$, were fitted to an established gastric emptying model to determine parameters for gastric emptying – tlag, t1/2, tlat tasc and Rmax. The percentage dose recovered (PDR) curves were presented to illustrate the standard enrichment after each preload. The time taken to reach maximal $^{13}CO_2$ excretion is the tlag, while the t1/2 is the time at which half of the label has been excreted in the breath. The latency time (tlat) is the initial delay in the cumulative $^{13}C$ excretion curve and the ascension time (tasc) is the time between the latency time and the half excretion time. Rmax is the maximum rate of label excretion.

## 3.3 Results

### 3.3.1 Subject characteristics

Thirty-two subjects were recruited from the general population. Two subjects withdrew from the study after completing only one visit: one withdrew due to time commitments and the other because of concerns raised by the media about the risks of participating in clinical research studies. These two subjects were replaced. Two subjects were excluded from the analysis after completing the procedures, one was found to have type 2 diabetes when the glucose samples were analysed and the other could not eat the entire preload meal and did not wish to eat the test meal. In total 28 subjects completed the study and met the eligibility criteria to be included in the analysis.

The characteristics of the study population are shown in Table 3.3.1. There were 19 female subjects and 9 male subjects. The mean age was 43.5 y (SD 16.6, range 19 – 68) and mean BMI was 22.8 kg/m$^2$ (SD 2.3, range 18 – 27.9 kg/m$^2$). There was no difference between male and female subjects in age ($p = 0.375$) or BMI ($p = 0.686$) but female subjects had a lower systolic ($p = 0.003$) and diastolic blood pressure ($p = 0.016$) than male subjects. The subjects who withdrew from the study did not differ from those who completed.

Body mass was measured at each visit (Table 3.3.2) and there were no significant changes over time.

**Table 3.3.1 Characteristics of the study population**

| n = 28 | Unit | Mean | SD | Range |
|---|---|---|---|---|
| **Age** | y | 43.5 | 16.6 | 19 – 68 |
| **Body mass** | kg | 66.3 | 8.65 | 45.6 – 85.8 |
| **Height** | m | 1.7 | 0.087 | 1.51 – 1.85 |
| **BMI** | $kg/m^2$ | 22.7 | 2.31 | 18 – 27.9 |
| **Systolic BP** | mmHg | 113 | 12 | 83 - 135 |
| **Diastolic BP** | mmHg | 66 | 10 | 47 - 86 |

**Table 3.3.2 Mean body mass of subjects at each visit (mean, 95% CI)**

None of the subjects changed in weight during the five visits (p=0.189).

| Meal | Mean body mass (kg) | 95% CI | | p value versus control |
|---|---|---|---|---|
| **Control** | 67.0 | 63.8, | 70.2 | |
| **Meat** | 66.6 | 63.4, | 69.8 | 0.105 |
| **Dairy** | 67.0 | 63.7, | 70.1 | 0.846 |
| **Nuts / legumes** | 67.2 | 64.0, | 70.4 | 0.442 |
| **Soy** | 66.9 | 63.7, | 70.1 | 0.601 |

### 3.3.2 Energy Intake

There was a significant difference in energy consumed at the test meal following the different preload meals ($p < 0.01$) (Table 3.3.3). Energy intake at the test meal was significantly lower following the soy meal than the control meal ($p = 0.02$). Energy intake after the meat, dairy, or nuts and legumes preload was not significantly different to the control. Energy intake after the soy preload was significantly lower than after all other preloads ($p = 0.005$ compared to control, $p = 0.02$ compared to meat, $p = 0.03$ compared to dairy, $p < 0.0001$ compared to nuts and legumes). The highest energy intake occurred after the nuts and legumes preload, where energy intake was significantly higher than the dairy ($p = 0.074$) and soy ($p = <0.0001$) preload but was not different from the control ($p = 0.2$) or meat preload ($p = 0.1$) (Figure 3.3.1).

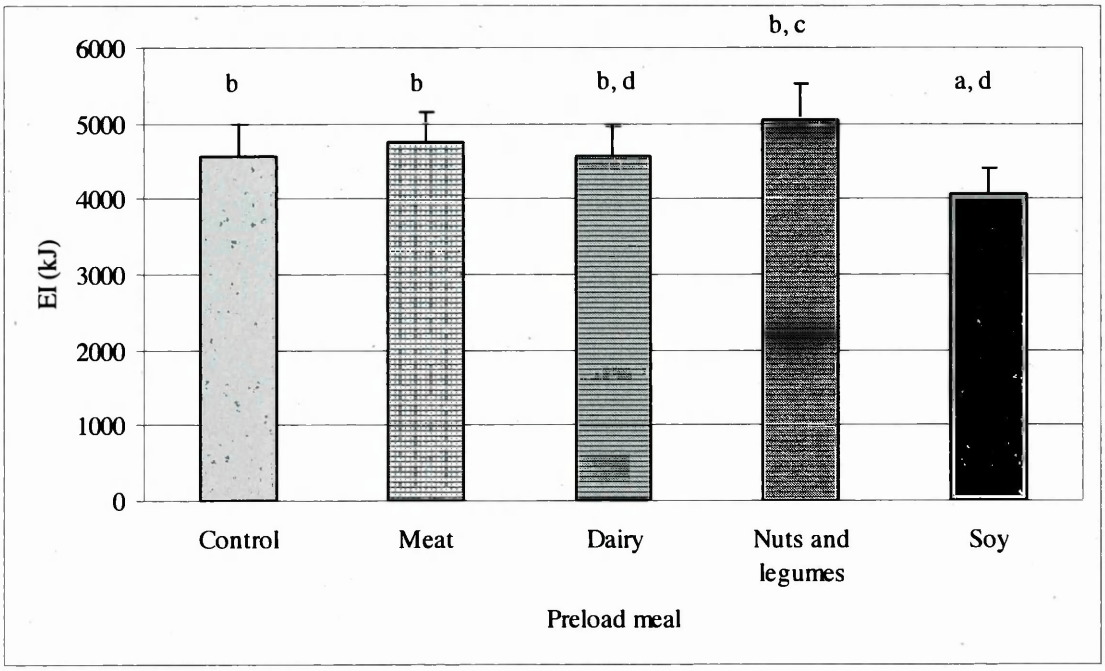
**Table 3.3.3 Energy intake at the test meal after each of the preload meals**

| Preload meal | Mean energy intake at the test meal (kJ) | Mean difference in energy intake versus control (kJ) | 95% CI of mean difference versus control | p value for difference versus control |
|---|---|---|---|---|
| **Control** | 4605 | | 3823, 5387 | |
| **Meat** | 4706 | 107 | -350, 563 | 0.647 |
| **Dairy** | 4560 | -35 | -491, 422 | 0.882 |
| **Nuts / legumes** | 5012 | 381 | -81, 843 | 0.106 |
| **Soy** | 4067 | -540 | -1000, -81 | 0.021* |

**Figure 3.3.1 Mean (SEM) energy intake (EI) at the test meal**

a, soy significantly lower than b, control, meat, dairy, nuts/legumes

c, nuts/legumes significantly higher than d, dairy and soy




Four subjects did not finish the control preload, two the meat preload, two the dairy preload, eight the nuts and legumes preload, and four could not finish the soy preload. In most cases only a small amount of food was left (10 – 50 g) but for the nuts and legumes preload, and the soy preload, the amount of food left was, in some cases, larger. The mean energy intake at the preload meal is shown in Table 3.3.4. The mean intake at the nuts and legumes preload was significantly less than the control preload ($p = 0.008$) and the time taken to consume the preload was lower in the meat ($p = 0.002$) and dairy preloads ($p = 0.004$).

**Table 3.3.4 Mean energy intake at the preload meal and the time taken to complete the meal.**

| Preload Meal | Mean energy intake at the preload meal (kJ) | SD | Mean time to consume the preload meal (min) | SD |
|---|---|---|---|---|
| **Control** | 2488 | 29 | 19.4 | 6.3 |
| **Meat** | 2491 | 39 | 16.5 | 3.5 |
| **Dairy** | 2487 | 49 | 16.7 | 4.1 |
| **Nuts / legumes** | 2410 | 229 | 19.3 | 6.6 |
| **Soy** | 2452 | 136 | 19.1 | 6.4 |
| **p value for difference between preload meals** | 0.02* | | < 0.001* | |

When the total energy consumed at the preload and test meals was analysed (Table 3.3.5) energy consumed on the soy-preload day was significantly less than the control day and was significantly lower than all other days ($p = 0.002$ versus control, $p = 0.04$ versus meat, $p = 0.01$ versus dairy, $p = 0.001$ versus nuts and legumes). The highest energy intake on the nuts and legumes experiment day was significantly greater than the soy day ($p = 0.001$) but not the dairy ($p = 0.4$), control ($p = 0.8$) or meat days ($p = 0.2$). The lower energy intake of the nuts and legumes preload reduced the total energy intake for the day and reduced the difference in total daily energy intake between the protein types.

One subject was observed packing some of the test meal to take home on the third visit and such confounding behaviour may have occurred on the previous two visits. When data from these visits was removed from the analysis there was no effect on the results and so this subject was kept in the group analysis.

**Table 3.3.5 Mean total daily energy intake (preload meal plus test meal).**

| Preload Meal | Mean total energy intake (preload plus test meal) (kJ) | Mean difference in energy intake versus control (kJ) | 95% CI of mean difference versus control | p value versus control |
|---|---|---|---|---|
| **Control** | 6931 | | 6107, 7756 | |
| **Meat** | 7203 | 271 | -261, 803 | 0.318 |
| **Dairy** | 7057 | 125 | -407, 658 | 0.644 |
| **Nuts / legumes** | 7275 | 344 | -188, 876 | 0.205 |
| **Soy** | 6374 | -557 | -1090, -25 | 0.040* |

### 3.3.3 Palatability

Palatability scores differed for the preload meals although absolute differences were small. The control preload scored higher than all the high-protein preloads for 'How sweet did you find the meal?' ($p=0.007$), 'How tasty did you find the meal?' ($p<0.001$), and 'How enjoyable did you find the meal?' ($p<0.001$).

The meat high-protein preload scored highest for 'How savoury did you find the meal?' ($p<0.001$), and 'How satisfying did you find the meal?' ($p<0.001$). The dairy preload scored lowest for 'How filling did you find the meal?' ($p=0.01$) and scores were slightly higher in response to the question 'How much more of this food do you think you could eat?'. The nuts and legumes preload scored lowest for 'How sweet did you find the meal?' ($p=0.007$), and for 'How savoury did you find the meal?' ($p<0.001$). The soy preload scored highest for 'How filling did you find the meal?' ($p=0.01$). Both the nuts and legumes preload and the soy preload scored lower than the other preloads for 'How tasty did you find the meal?' ($p<0.001$), 'How pleasant did you find the meal?' ($p = 0.0001$), 'How satisfying did you find the meal?' ($p<0.001$) and 'How enjoyable did you find the meal?' ($p<0.001$).

The test meal was perceived to be less sweet ($p = 0.04$) and more savoury ($p = 0.02$) after the nuts and legumes preload than after the other preload meals. The test meal did not differ in scores for 'tasty', 'pleasant', 'filling', 'satisfying', 'enjoyable' or how much more food could be eaten.

### 3.3.4 Hunger and satiety

Hunger was significantly lower at $t = 30$ min after the soy preload ($p = 0.008$) and at $t = 90$ min after the nuts and legumes preload ($p = 0.045$). At $t = 180$ there was a non-significant reduction in hunger after the soy preload ($p = 0.083$). There was no difference between the groups in any other parameters for the question 'How hungry are you?' (Figure 3.3.2 and Figure 3.3.3). For the question 'How full do you feel' (Figure 3.3.4 and Figure 3.3.5) the score at $t = 180$ min after the soy preload was significantly higher than the other preloads ($p = 0.018$). The subsequent energy intake at the test meal after the soy

preload was then significantly lower (Figure 3.3.1). There was no difference in any parameters for the question 'How strong is your desire to eat?' (Figure 3.3.6 and Figure 3.3.7). In response to the question 'How much food do you think you could eat?' (Figure 3.3.8 and Figure 3.3.9) scores were significantly lower at t = 90 min after the nuts and legumes preload and the soy preload ($p = 0.0496$) and the time to the minimum score was less after the nut and legumes preload ($p = 0.015$).

An appetite score was calculated as the mean response to 'hunger', 'fullness' (and reciprocal of the fullness score), 'desire to eat', and 'amount that could be eaten' (Bowen *et al.*, 2006c). There was no difference in any parameters in the appetite score between study preloads.

Other factors were included in the questionnaire, mainly as a diversion from the appetite questions above. Some differences between preload meals were observed. In response to the question 'How content are you?' (Figure 3.3.10 and Figure 3.3.11) there were significant differences in scores during the investigation days. Subjects were more content at baseline prior to the nuts and legumes preload ($p = 0.006$) and meat preload ($p = 0.012$). At 30 min contentment remained high after the meat preload but had fallen after the nuts and legumes preload and was lower after the soy preload ($p = 0.038$). There was no difference in scores between 30 and 180 min but at 180 min there was a trend for lower scores after the nuts and legumes preload and higher for the meat preload ($p = 0.059$). AUC for the entire investigation day (t = 0 - 300 min) was higher for the meat preload and lower for the nuts and legumes preload ($p = 0.021$) but there was no difference between the AUC for the groups between the preload and test meals (t = 0 – 180 min).

'Irritability' scores (Figure 3.3.12 and Figure 3.3.13) were generally low but were lower at baseline prior to the meat preload ($p = 0.033$) and the nuts and legumes preload ($p = 0.011$) which were not affected by order of visit. Scores remained low after the meat preload at 150 min ($p = 0.017$) and were lower after the dairy preload than the control ($p = 0.035$). The maximum score after the meat preload was lower than after the other preloads ($p = 0.035$) and AUC after the meat preload was lower between the preload and test meals ($p = 0.027$) and throughout the entire investigation day ($p = 0.034$).

In response to the question 'How depressed are you?' (Figure 3.3.14 and Figure 3.3.15) the only difference in any of the parameters was the maximum score after the meat preload was higher than after the other preloads ($p = 0.029$). The scores for the question 'How alert are you?' (Figure 3.3.16 and Figure 3.3.17 were lower after the nuts and legumes preloads ($p = 0.004$) and soy preload ($p = 0.013$) at 180 min but there were no differences for any other parameter.

**Figure 3.3.2 Mean VAS score for the question 'How hungry are you?'**

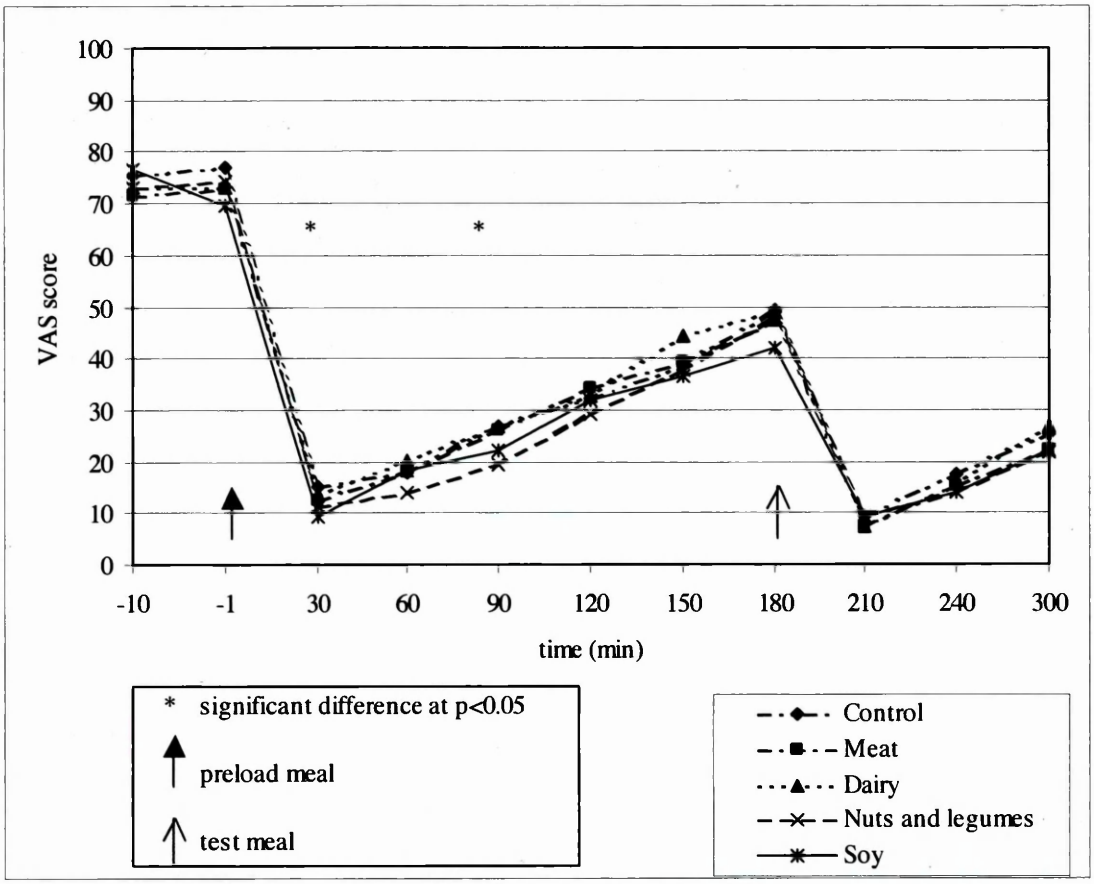



**Figure 3.3.3 Mean AUC for VAS scores for the question 'How hungry are you?' from baseline until the test meal at 180 min**

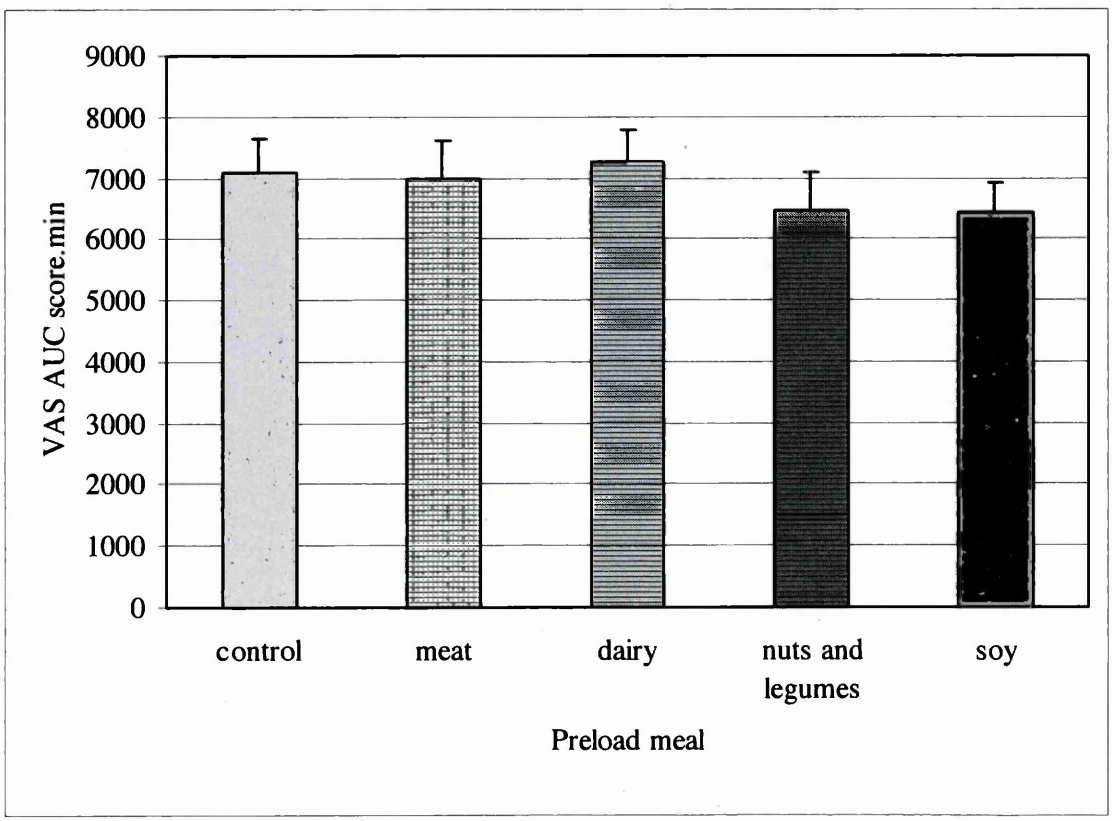


**Figure 3.3.4 Mean VAS score for the question 'How full do you feel?'**

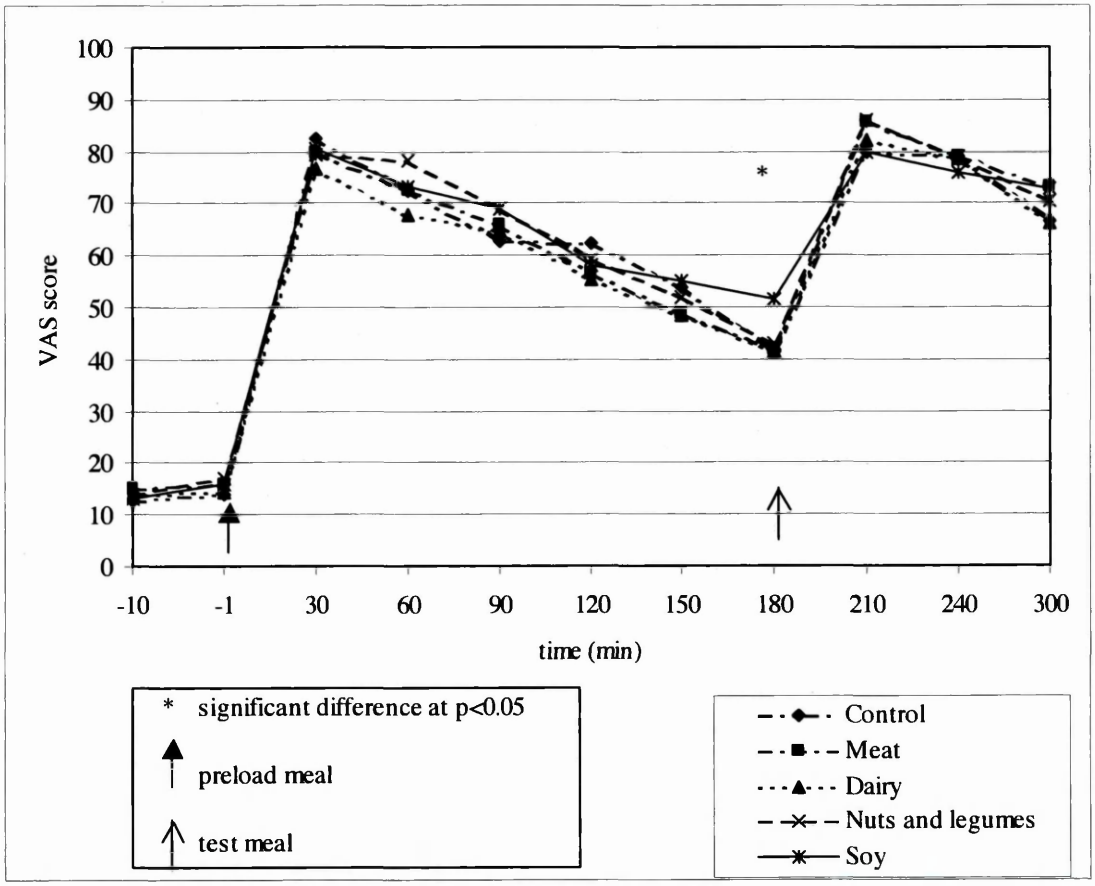



**Figure 3.3.5 Mean AUC for VAS scores for the question 'How full do you feel?' from baseline until the test meal at 180 min**

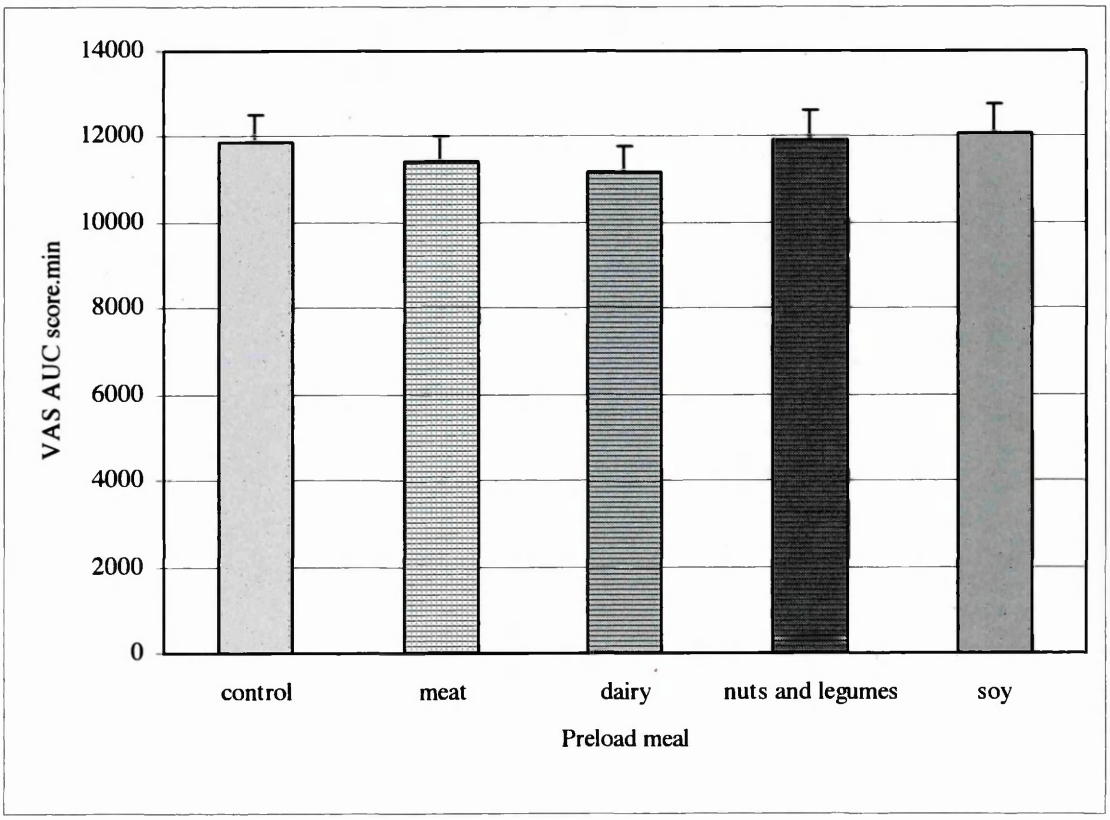


**Figure 3.3.6 Mean VAS score for the question 'How strong is your desire to eat?'**

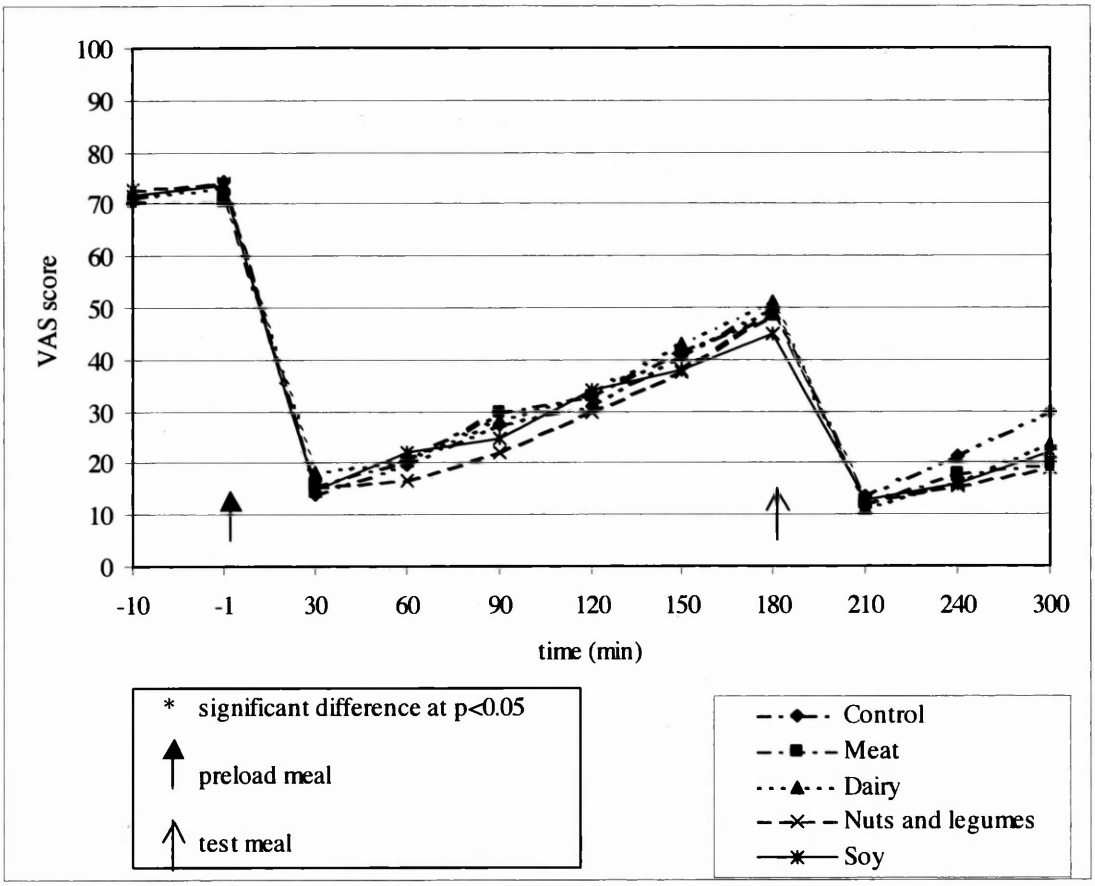



**Figure 3.3.7 Mean AUC for VAS scores for the question 'How strong is your desire to eat?' from baseline until the test meal at 180 min**

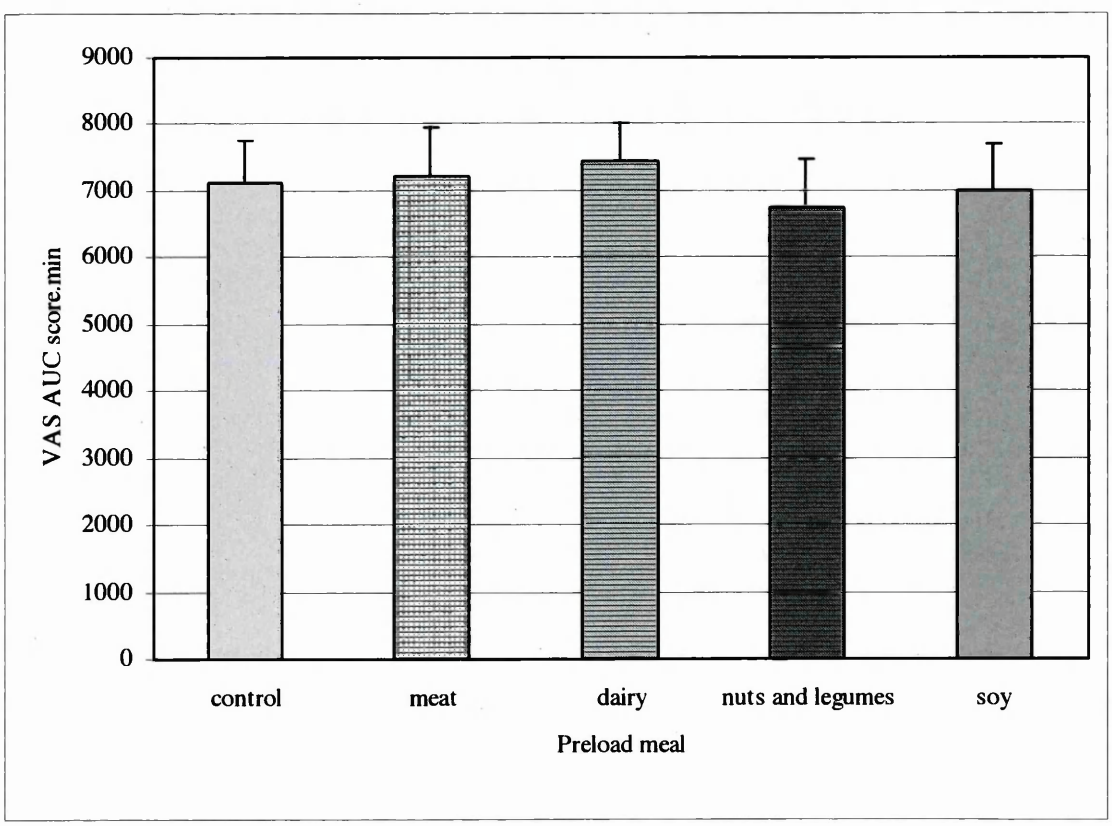


**Figure 3.3.8 Mean VAS score for the question 'How much food do you think you could eat?'**

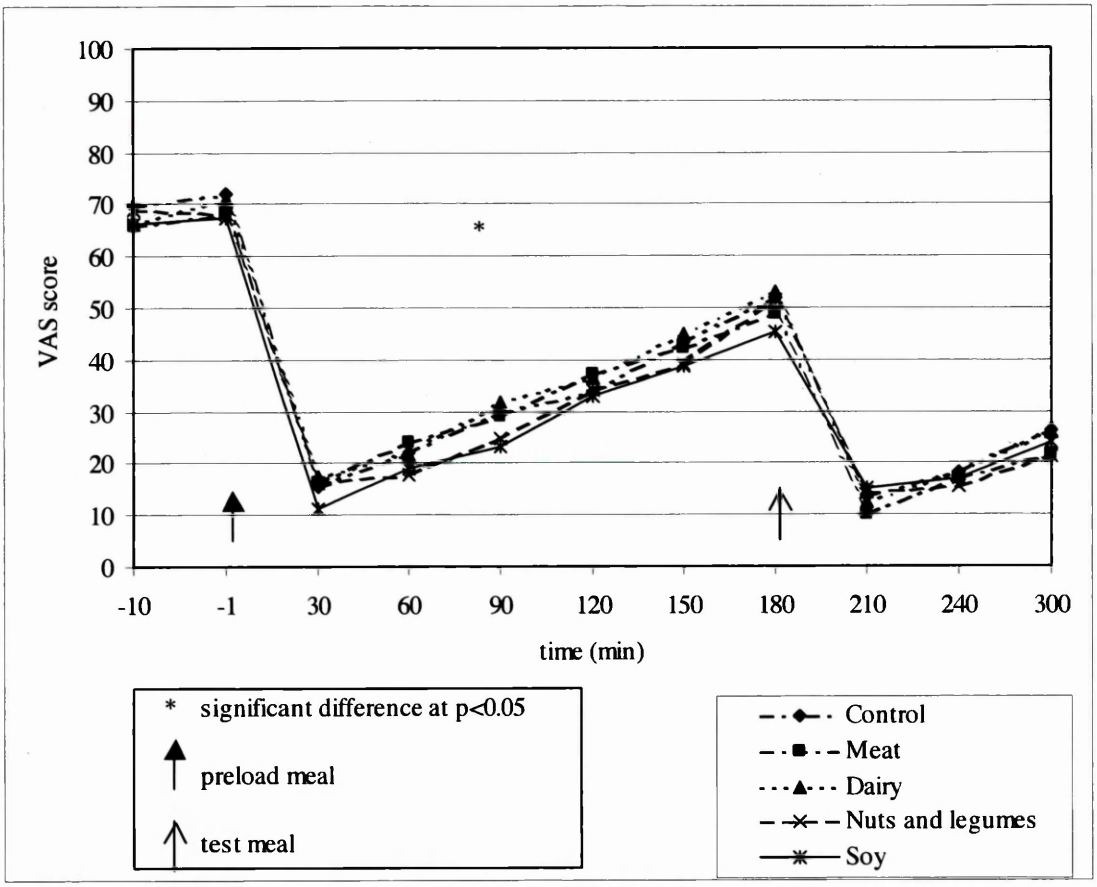



**Figure 3.3.9 Mean AUC for VAS scores for the question 'How much food do you think you could eat?' from baseline until the test meal at 180 min**

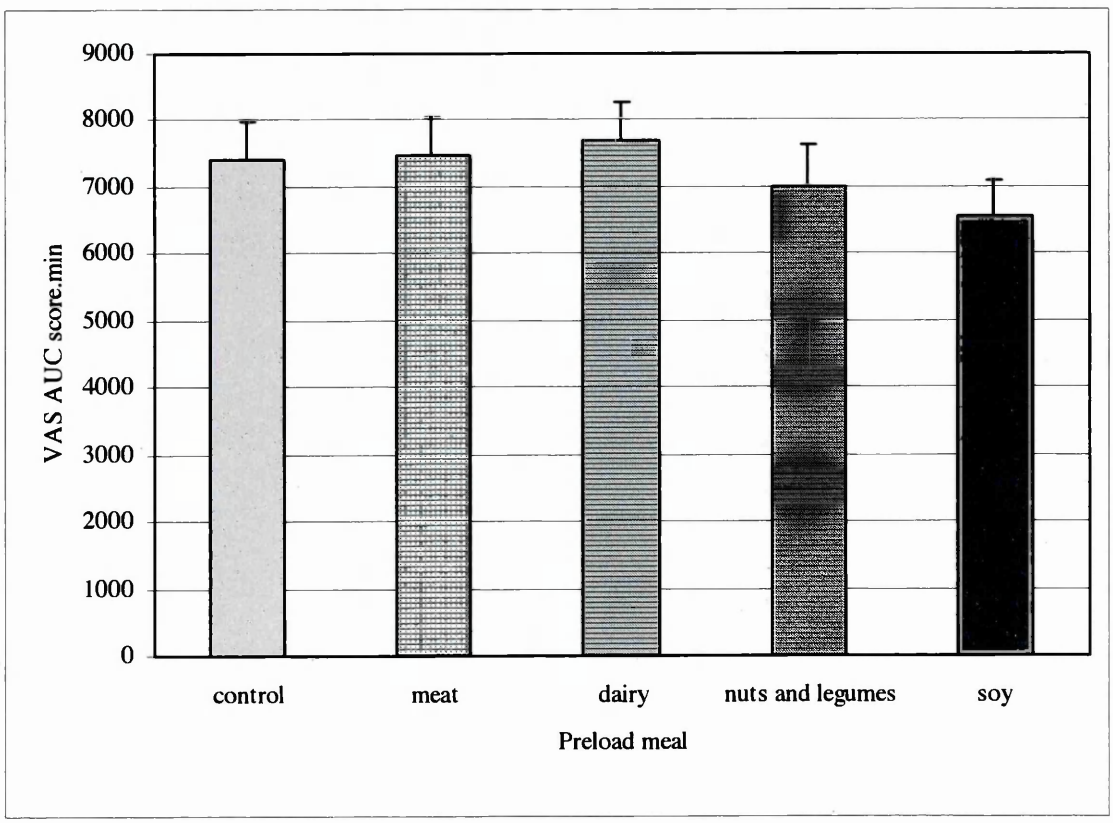


**Figure 3.3.10 Mean VAS score for the question 'How content are you?'**

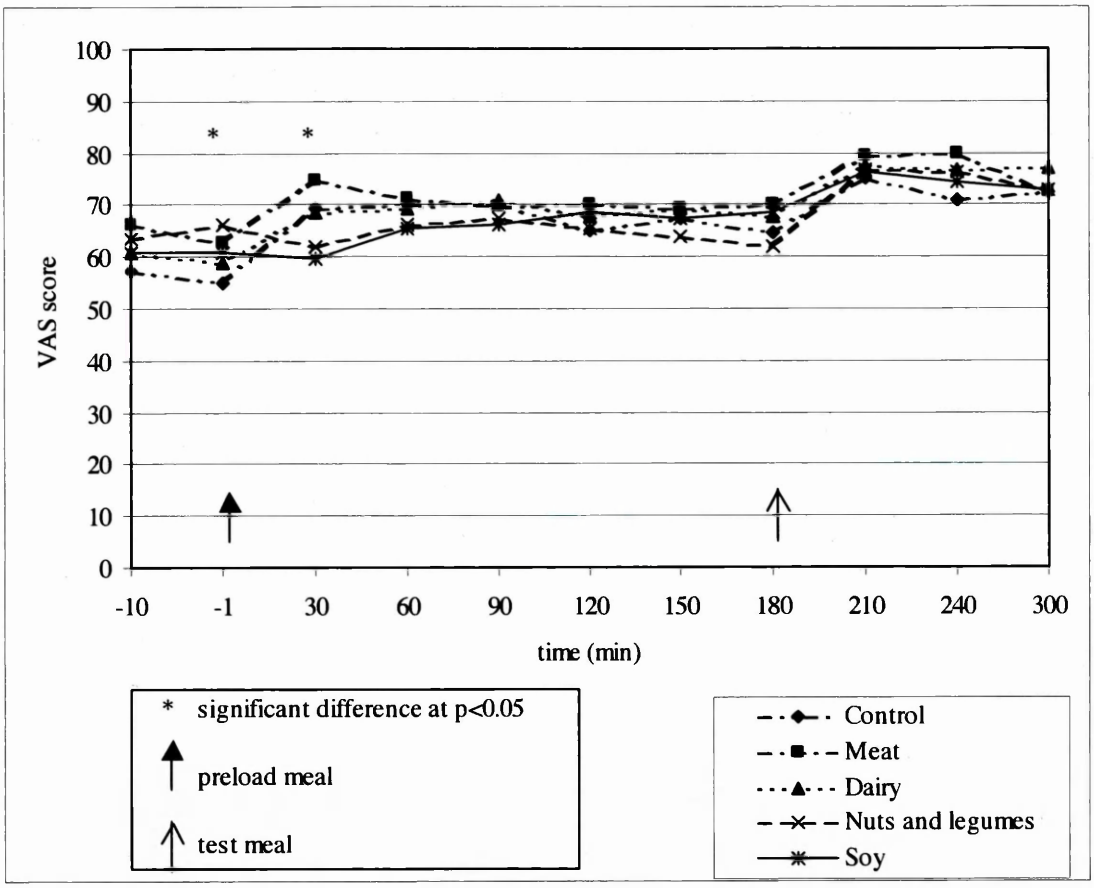



**Figure 3.3.11 Mean AUC for VAS scores for the question 'How content are you?' from baseline until the test meal at 180 min**

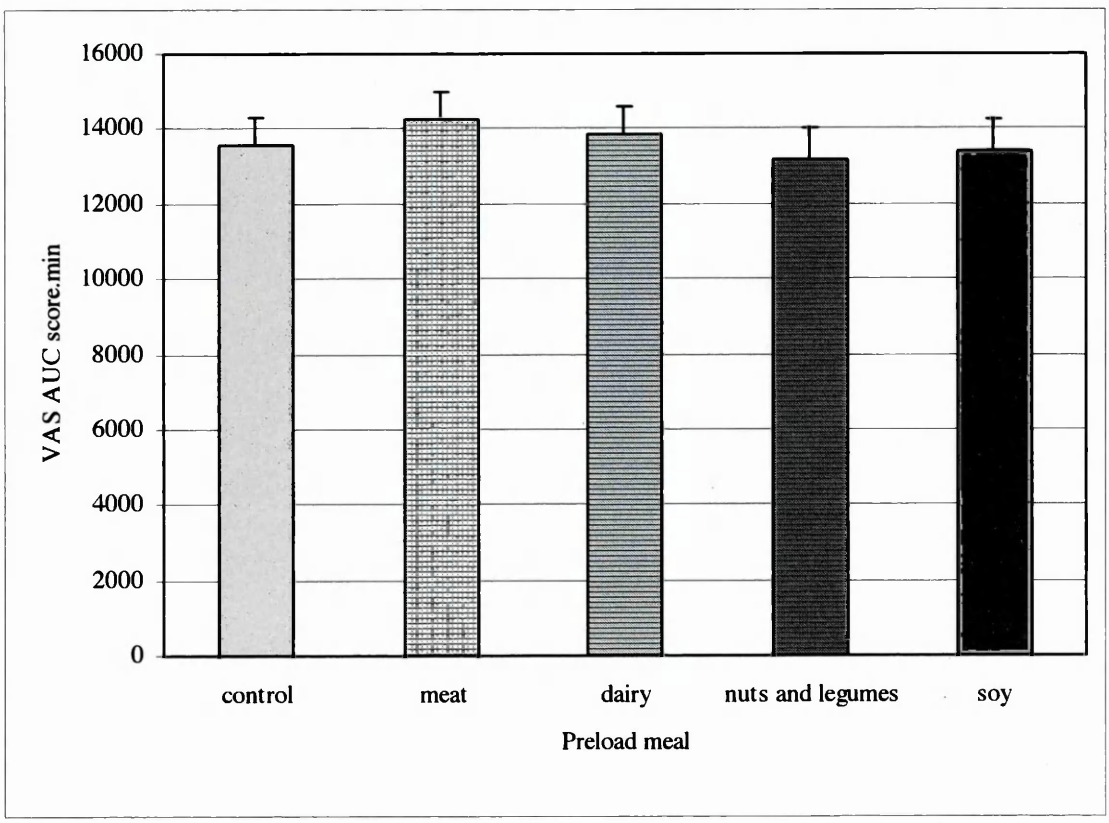


**Figure 3.3.12 Mean VAS score for the question 'How irritable are you?'**

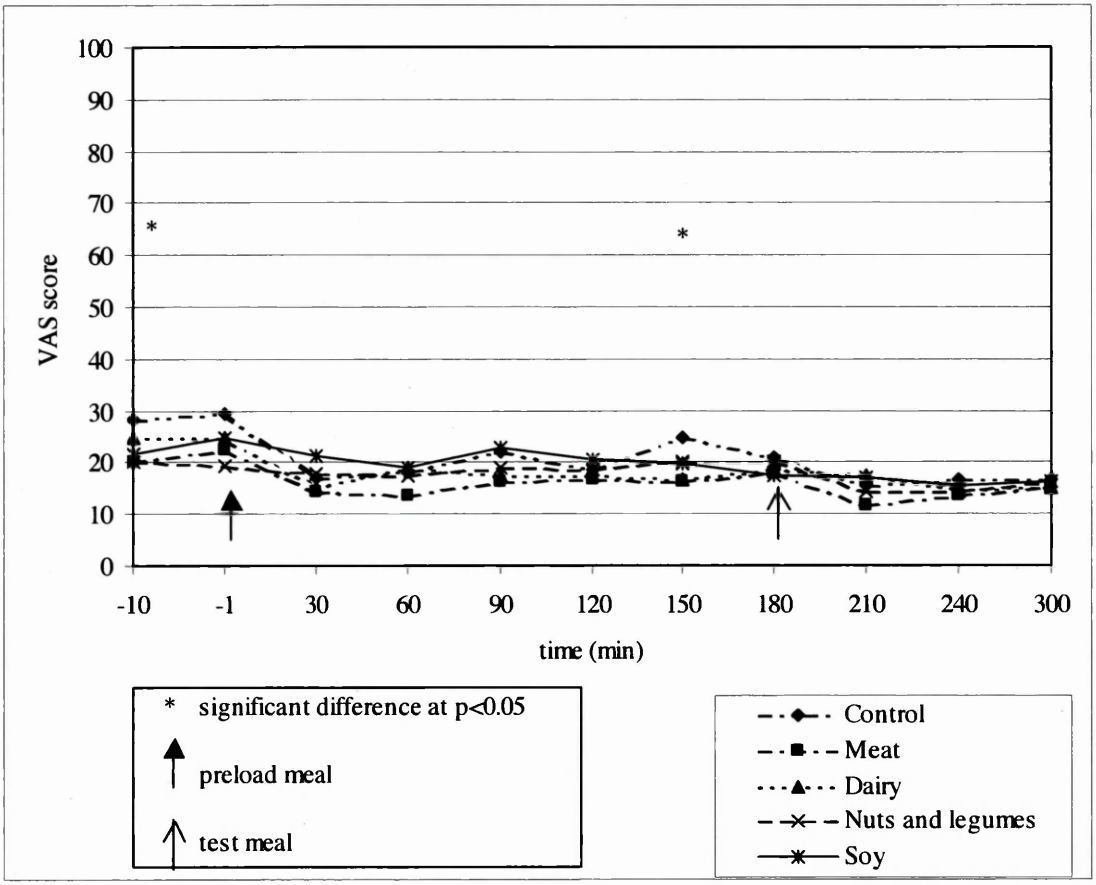



**Figure 3.3.13 Mean AUC for VAS scores for the question 'How irritable are you?' from baseline until the test meal at 180 min**

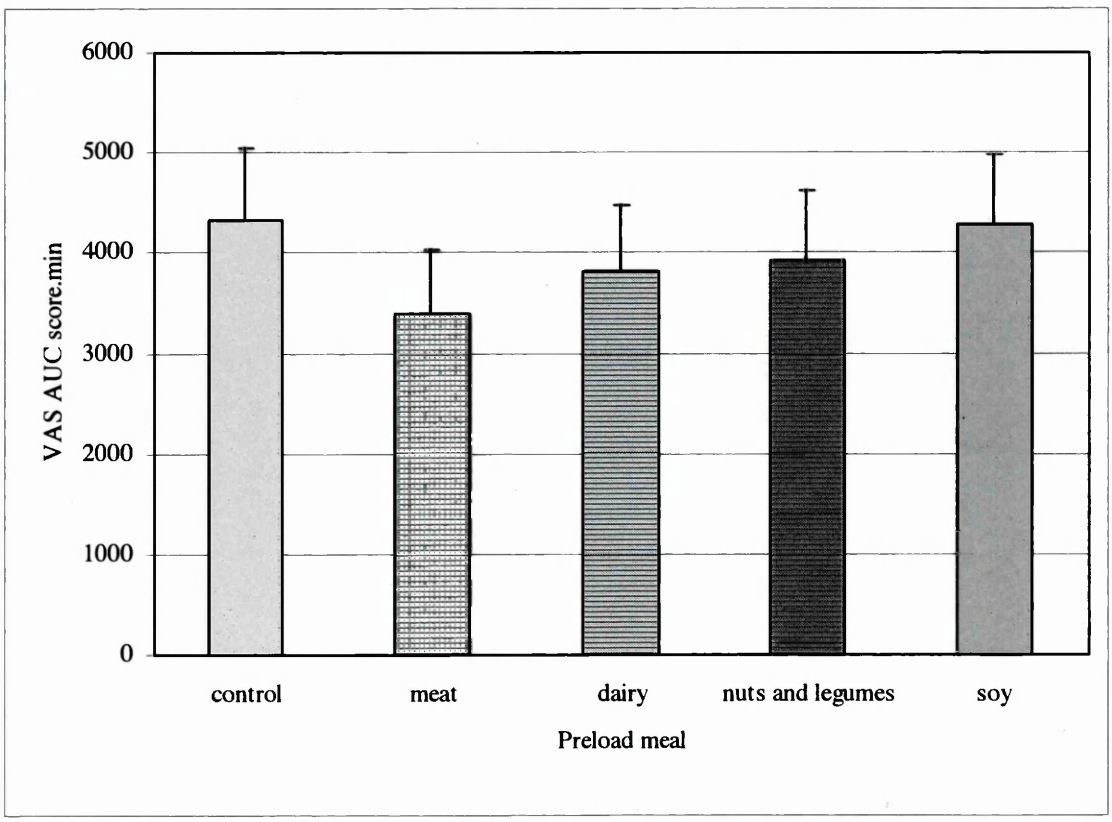


**Figure 3.3.14 Mean VAS score for the question 'How depressed are you?'**

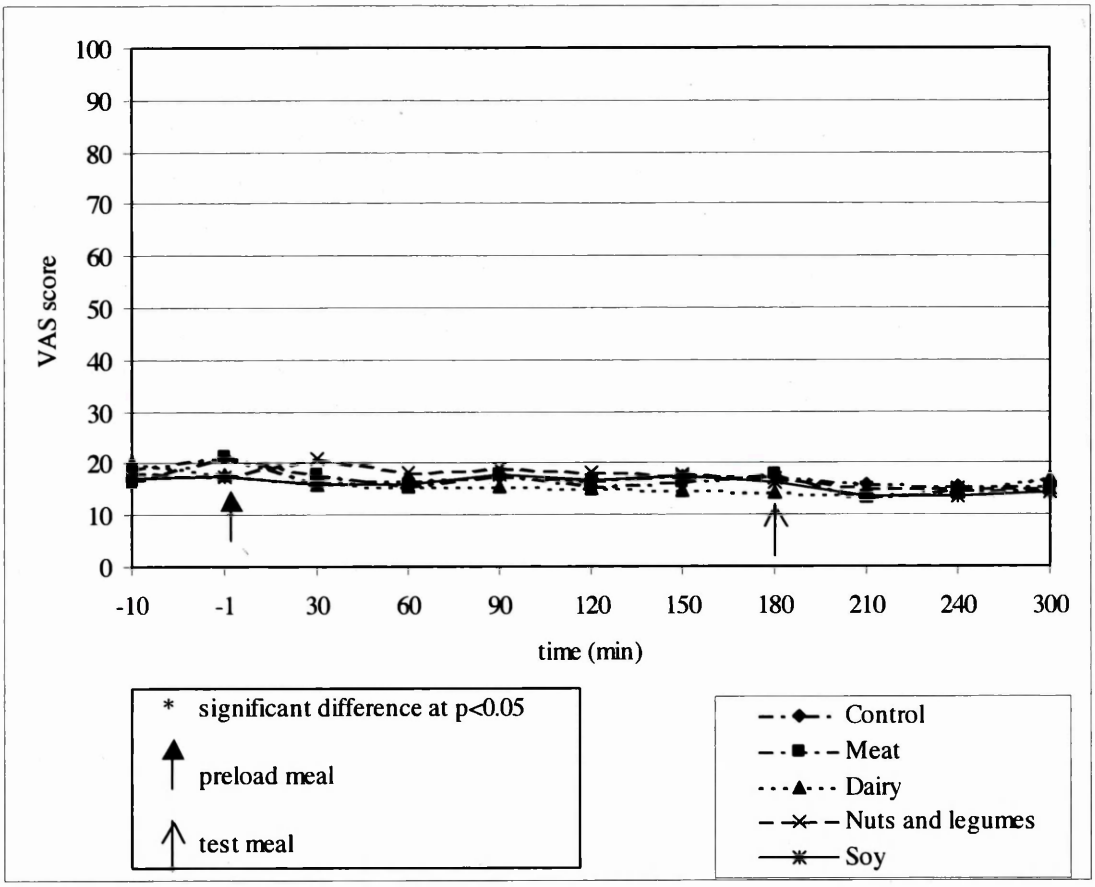



**Figure 3.3.15 Mean AUC for VAS scores for the question 'How depressed are you?' from baseline until the test meal at 180 min**

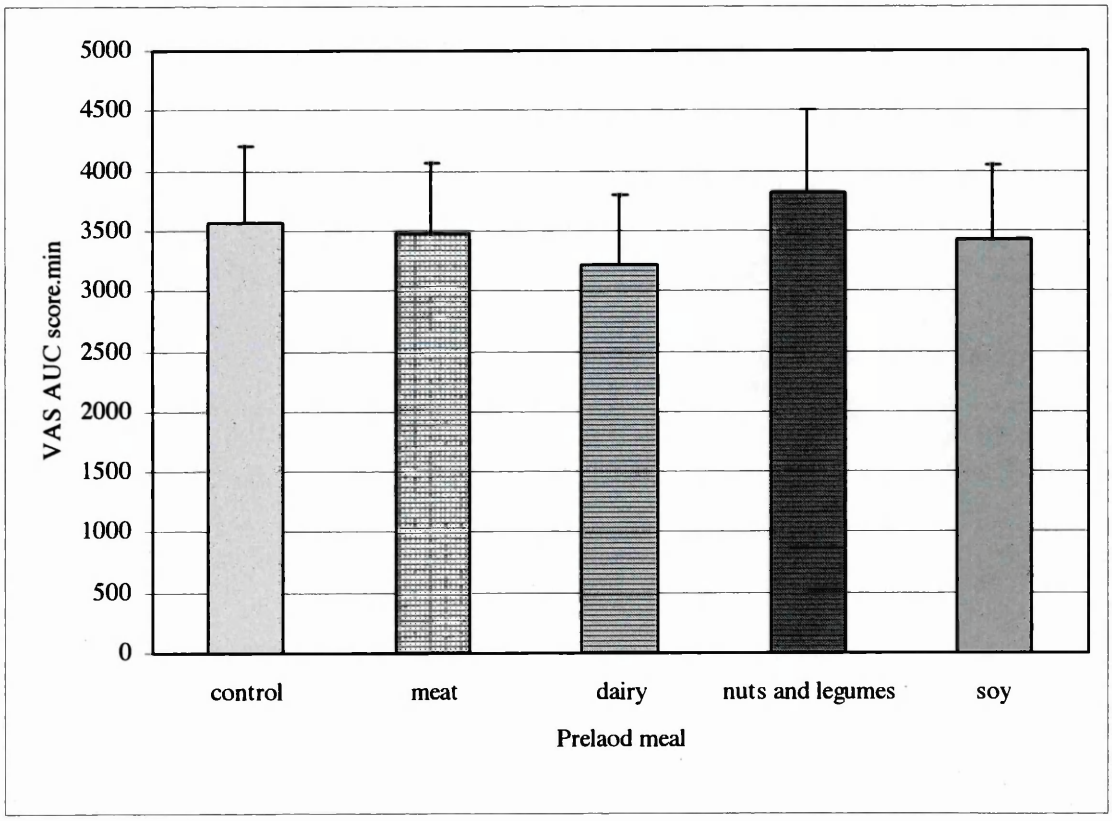


**Figure 3.3.16 Mean VAS score for the question 'How alert are you?**

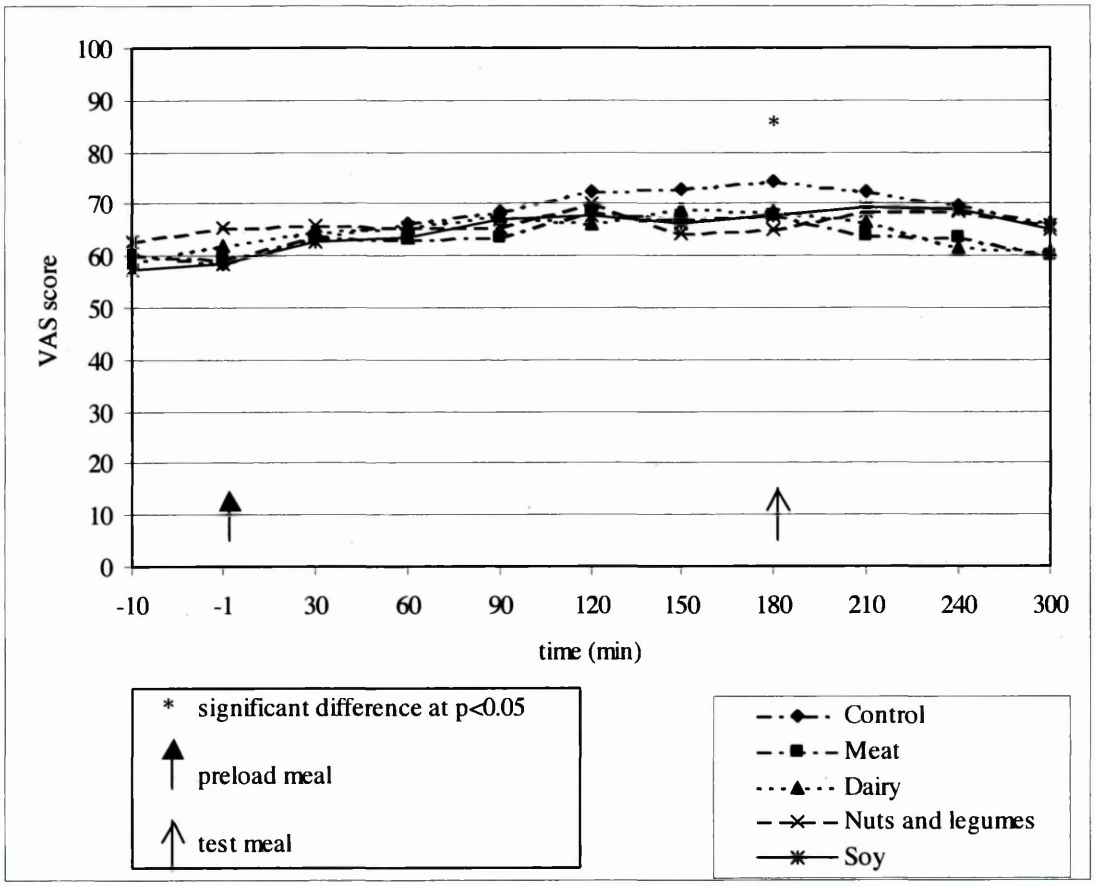
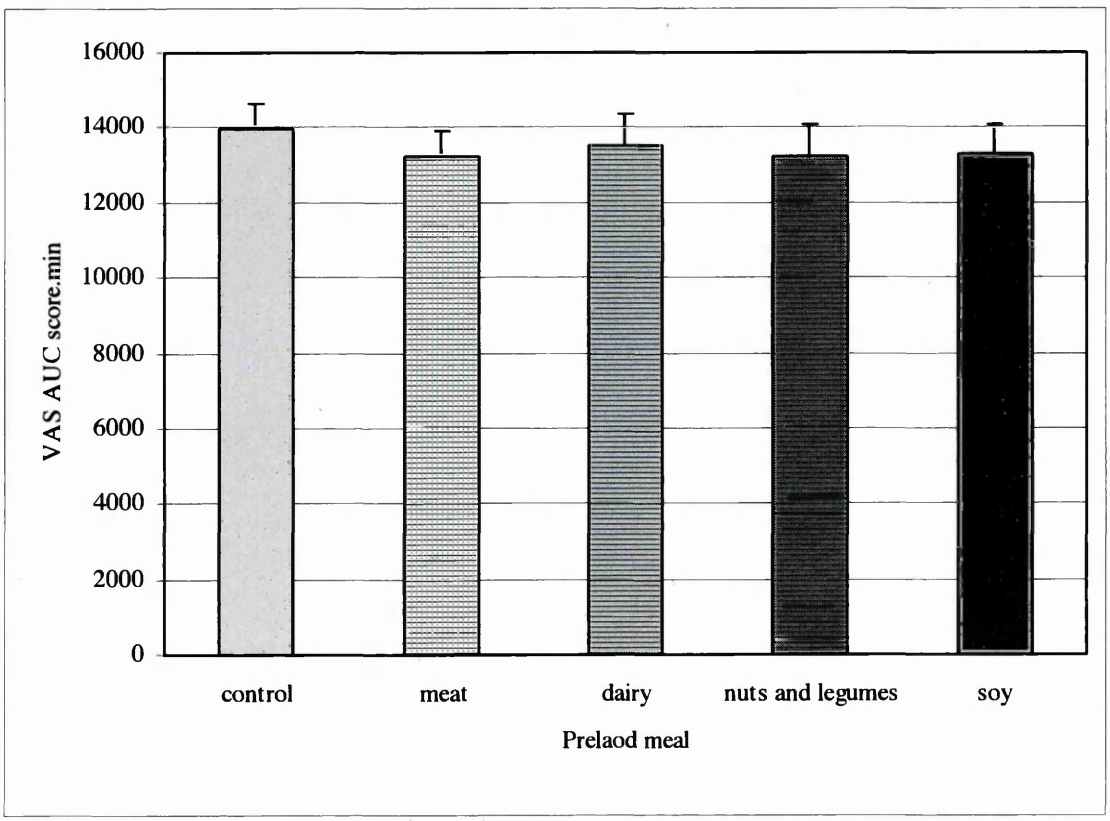



**Figure 3.3.17 Mean AUC for VAS scores for the question 'How alert are you?' from baseline until the test meal at 180 min**



### 3.3.5 Blood markers of metabolic risk and satiety

Mean blood glucose at baseline did not differ between preload meals, but curves following the preload were different at all time points (Figure 3.3.18). The glucose curve after the nuts and legumes preload was the flattest with the lowest peak at t = 30 min ($p < 0.001$) and the smallest post-prandial dip at t = 90 min ($p < 0.001$). The highest peak was after the control preload followed by the dairy and meat preloads, then the soy and nuts and legumes preloads ($p < 0.00001$). The greatest post-prandial dip was after the dairy preload ($p < 0.001$) and glucose levels remained significantly lower throughout the whole post-prandial period. Prior to the test meal (t = 180 min) the glucose level was highest after the nuts and legumes preload, followed by the soy, meat, dairy and control preloads ($p < 0.001$).

AUC was analysed using the two methods as discussed in Chapter 2. AUC was lowest after the dairy preload and highest after the nuts and legumes preload ($p < 0.001$) (Figure 3.3.19). Given the large post-prandial dip, analysis of AUC was challenging, as the recommended methods ignore or remove parts of the curve below baseline (Brouns, 2005) thus omitting any effects of post-prandial excursions on hunger and metabolic risk.

**Figure 3.3.18 Mean blood glucose concentration after the preload meals**

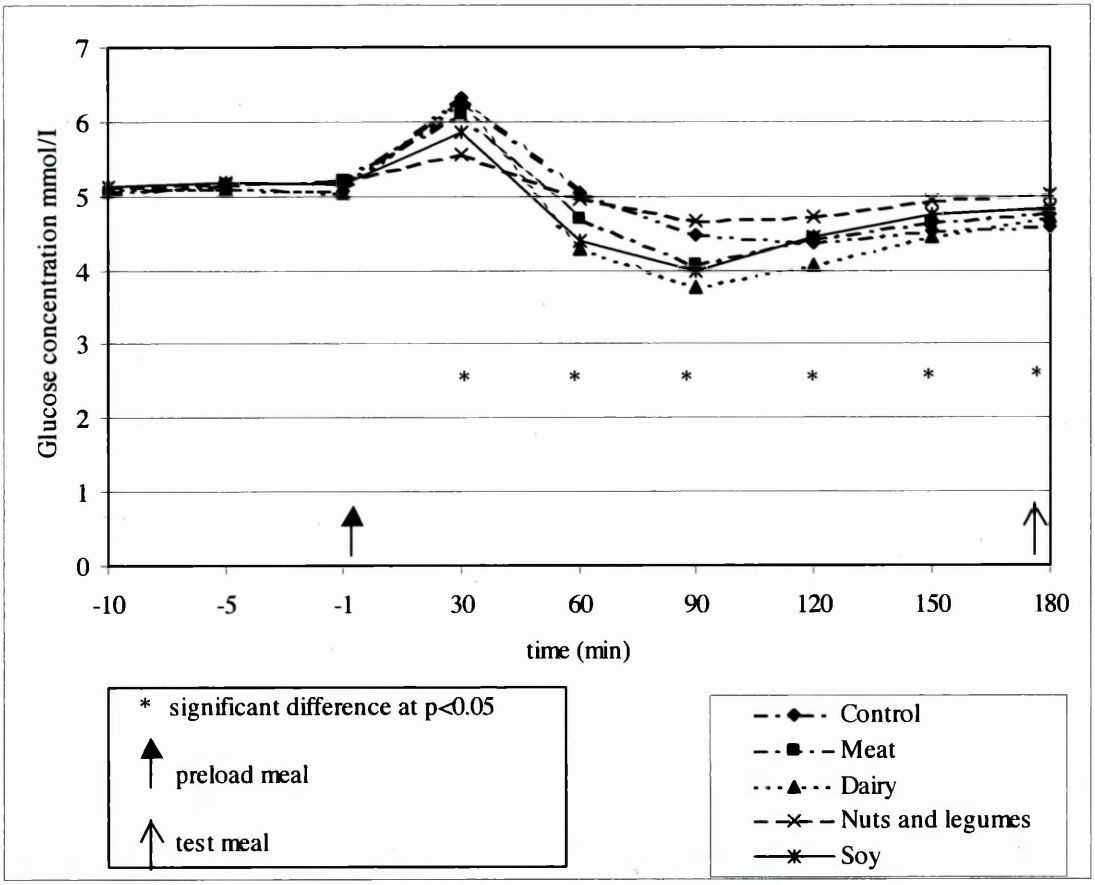



**Figure 3.3.19 Mean AUC for glucose concentration from baseline until the test meal at 180 min**

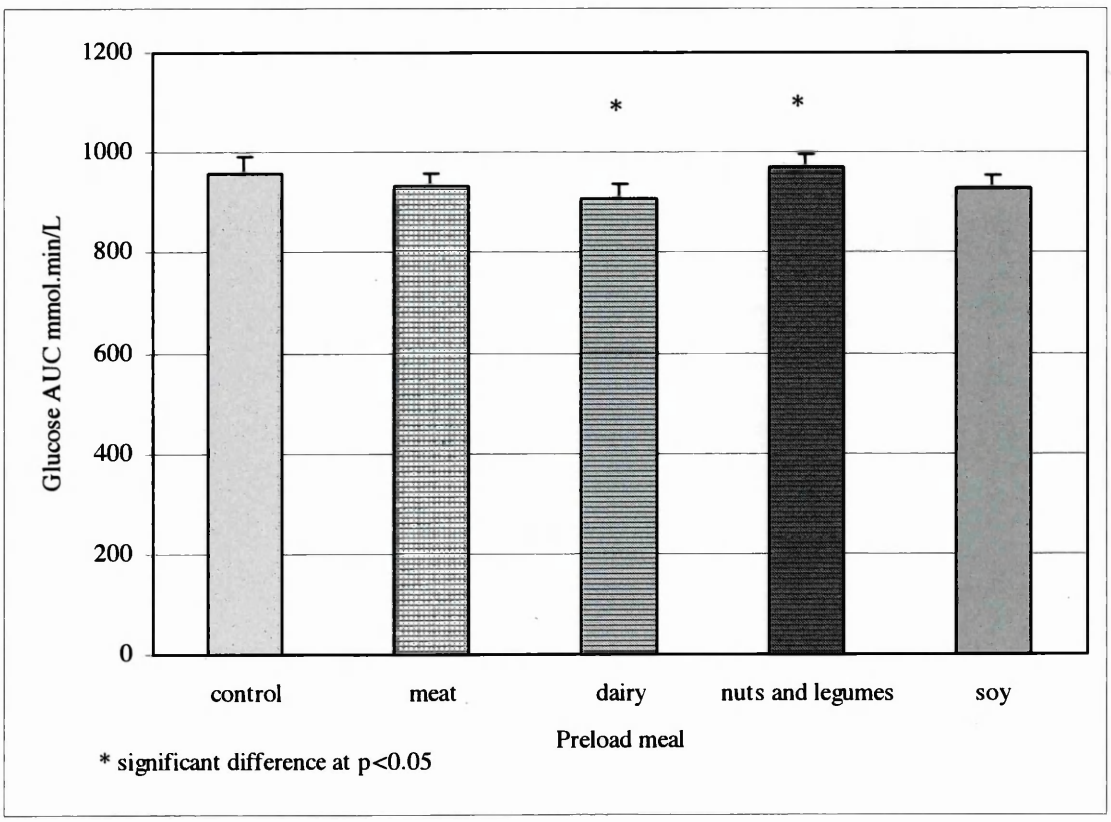


Figure 3.3.20 shows the blood insulin curves following the preload meals. There was no difference at baseline, then levels peaked between 30 and 60 min, with significantly higher levels after the dairy, control and meat preload, and low levels after the soy and nuts and legumes preloads ($p < 0.001$) (in the same order as the peak glucose concentration). By t = 180 min there was no difference between preloads although the insulin levels had not returned to baseline. AUC was significantly different between preloads, with the control having the highest AUC, followed by the dairy, meat, soy, and nuts and legumes preload ($p<0.001$) (Figure 3.3.21).

NEFA concentration did not differ at baseline and fell after all preloads following the same pattern (Figure 3.3.22). NEFA levels remained higher after the nuts and legumes preload at t = 60 ($p < 0.00001$), 90 ($p = 0.0002$), and 120 min ($p = 0.022$) but by t = 180 min the highest NEFA concentration was after the soy preload followed by the nuts, meat, dairy and control preloads ($p = 0.0263$). AUC was significantly higher after the nuts and legumes preload using the first AUC model ($p = 0.005$) (Figure 3.3.23). The second model was not used as all the area below the baseline is subtracted from the area above the baseline, and so is not informative when concentrations fall from baseline and do not subsequently rise.

**Figure 3.3.20 Mean blood insulin concentration after the preload meals**

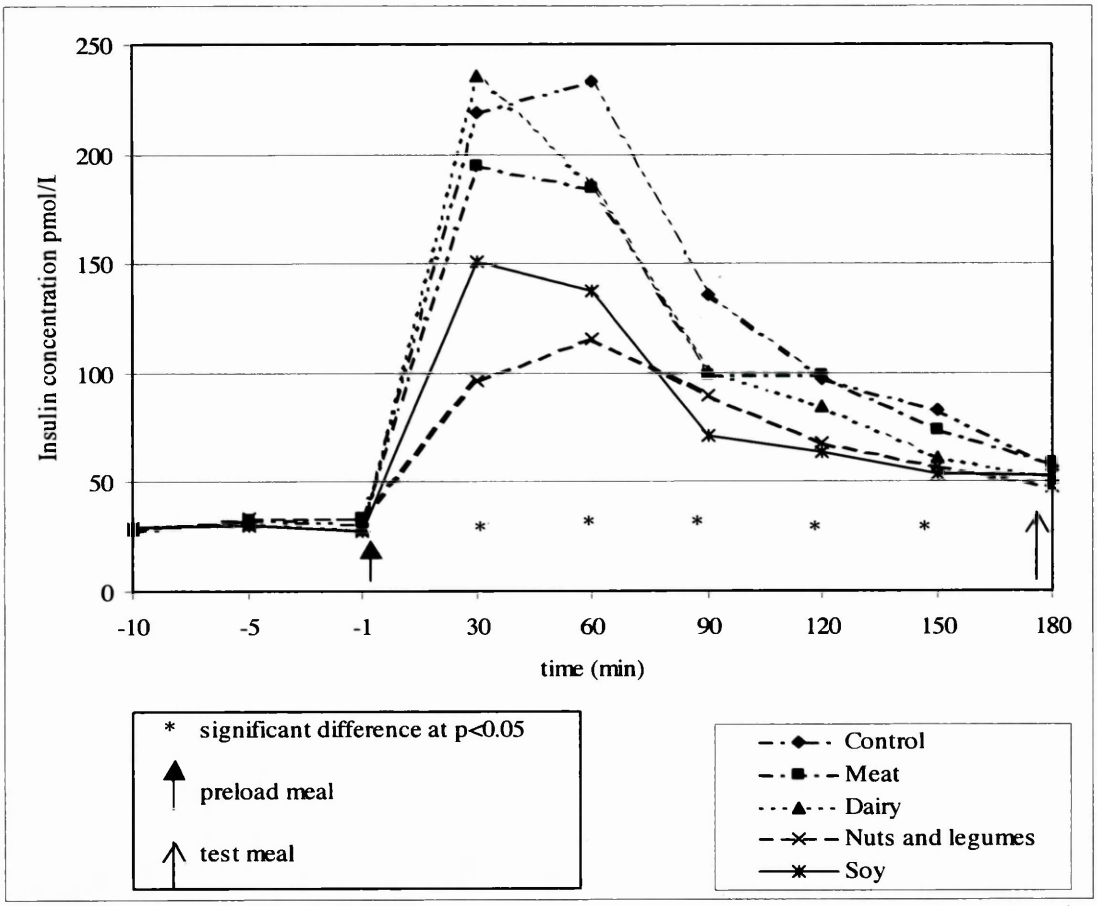



**Figure 3.3.21 Mean AUC for blood insulin concentration from baseline until the test meal at 180 min**

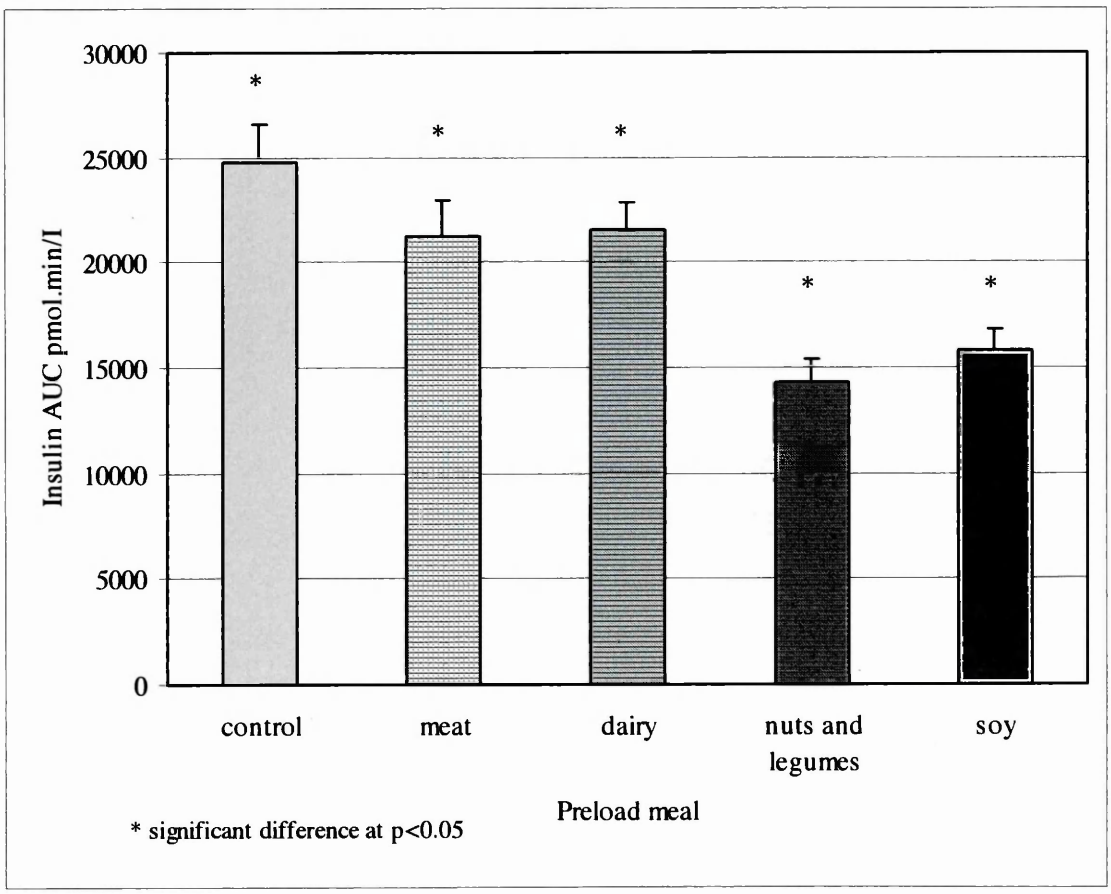


**Figure 3.3.22 Mean blood NEFA concentration after the preload meals**

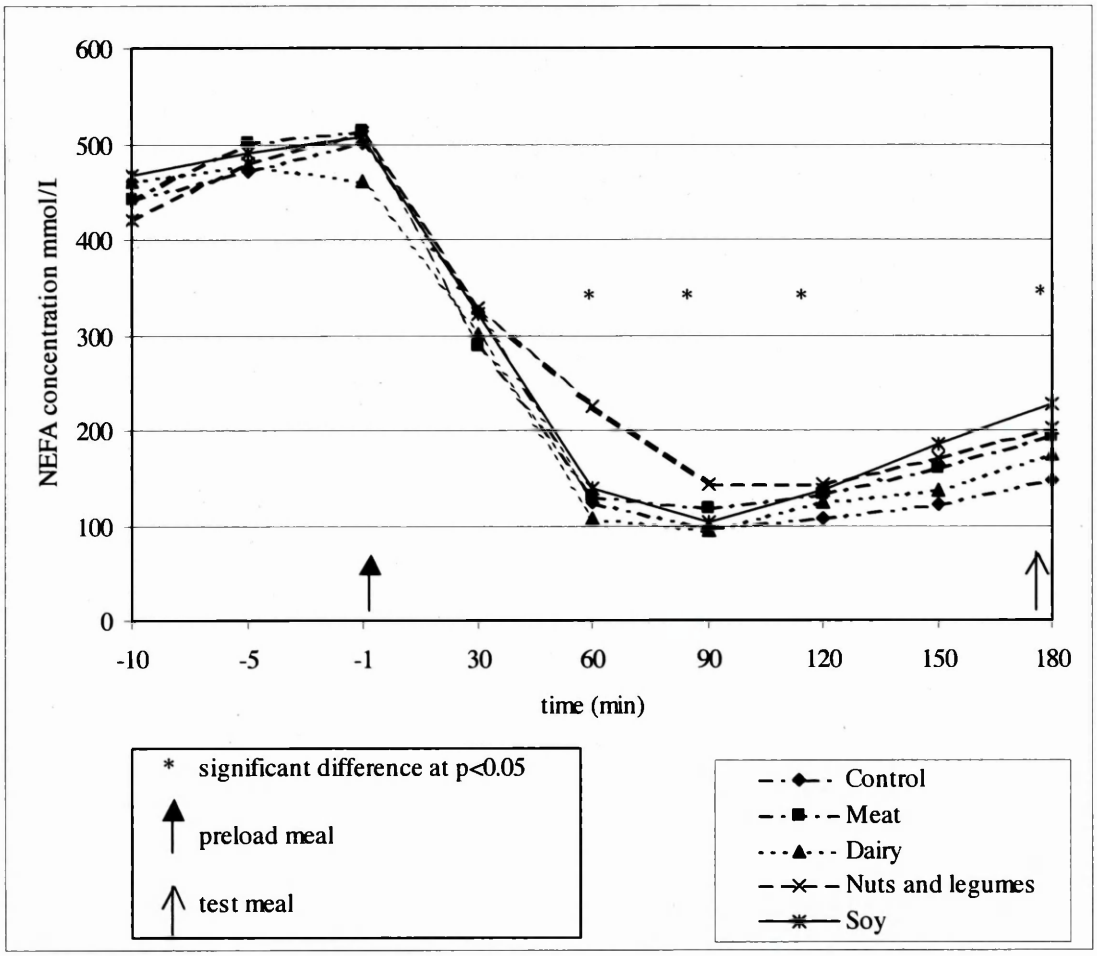
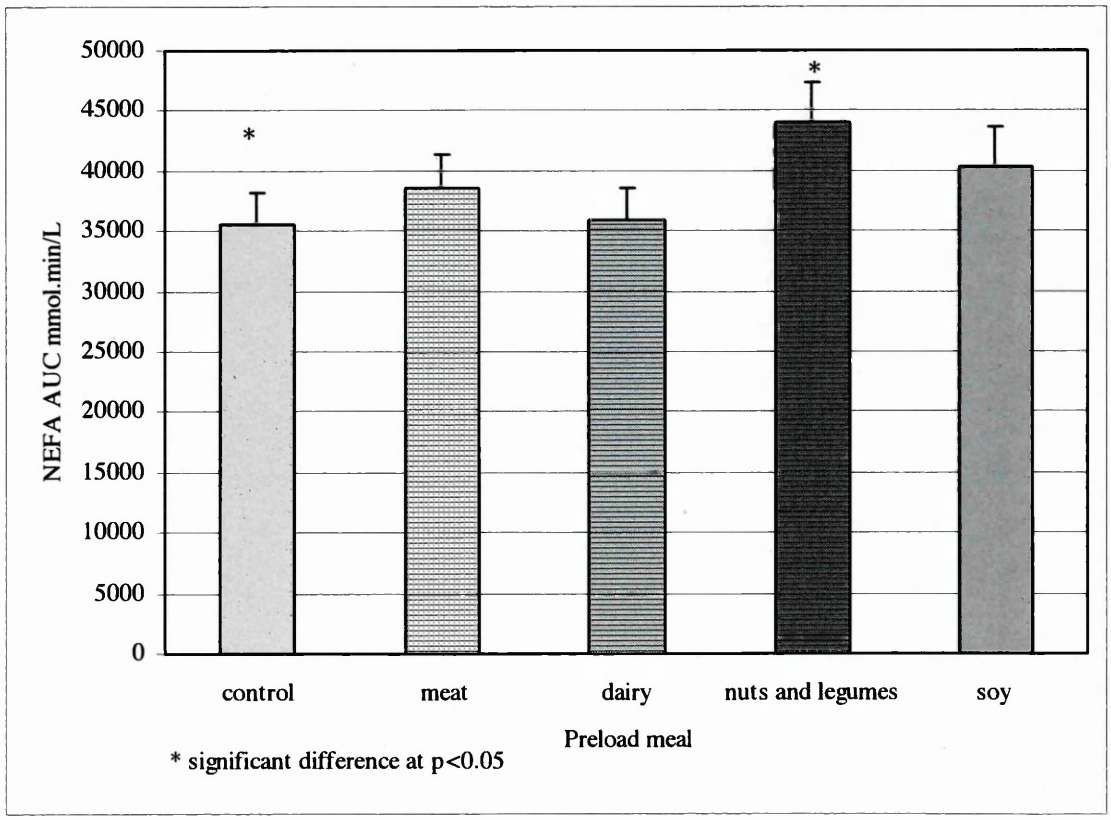



**Figure 3.3.23 Mean AUC for NEFA concentration from baseline until the test meal at 180 min**



### 3.3.6 Gastric Emptying

The mean PDR (percentage dose recovered) was higher after the soy preload at 49 ± 11 % compared with 45 ± 7% for the control and 45 ± 10 % for the other preloads. Figure 3.3.24 shows the mean standard enrichment curve after each preload. Table 3.3.6 shows the mean direct gastric emptying parameters for the five preload meals.

**Figure 3.3.24 $^{13}C$-isotopic enrichment in breath $CO_2$**

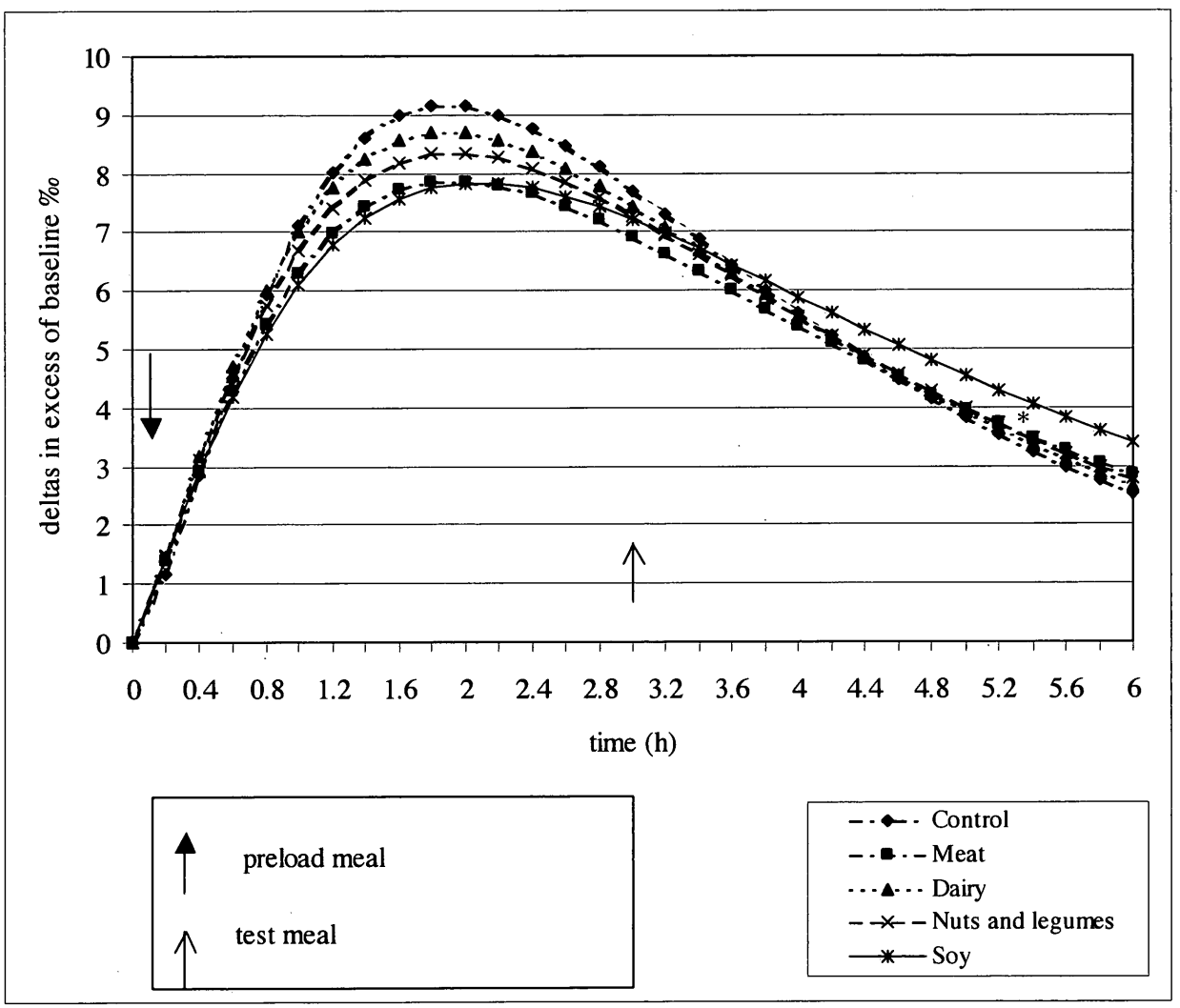


**Table 3.3.6 Gastric emptying direct parameters (mean ± sd)**

| Preload Meal | Tlag (h) | t1/2 (h) | tlat (h) | tasc (h) | Rmax(out) ($h^{-1}$) |
|---|---|---|---|---|---|
| **Control** | 1.92 ± 0.25 | 2.94 ± 0.43 | 0.68 ± 0.10 | 2.26 ± 0.39 | 0.23 ± 0.04 |
| **Meat** | 2.00 ± 0.35 | 3.27 ± 0.65 | 0.64 ± 0.11 | 2.63 ±0.59 | 0.20 ± 0.05 |
| **Dairy** | 1.89 ± 0.32 | 2.99 ± 0.47 | 0.64 ± 0.14 | 2.35 ± 0.38 | 0.22 ± 0.04 |
| **Nuts / legumes** | 1.94 ± 0.36 | 3.10 ± 0.52 | 0.64 ± 0.15 | 2.46 ± 0.41 | 0.21 ± 0.04 |
| **Soy** | 2.10 ± 0.34 | 3.47 ± 0.66 | 0.65 ± 0.12 | 2.81 ± 0.61 | 0.19 ± 0.06 |
| **p value between preload meals** | $p = 0.003$ | $p < 0.001$ | $p = 0.58$ | $p < 0.001$ | $p < 0.001$ |

Lag time (tlag), the time taken to reach maximal $^{13}CO_2$ excretion, was significantly longer after the soy preload, at 2.10 ± 0.34 h ($p = 0.002$) than the other preloads. The half excretion time (t1/2), the time taken for 50 % of the total $^{13}CO_2$ excreted in the breath to appear, was significantly longer after the soy preload (3.47 ± 0.66 h, $p < 0.0001$) and meat preload (3.27 ± 0.65 h, $p = 0.001$), followed by the nuts and legumes preload (3.10 ± 0.52 h), dairy preload (2.99 ± 0.47 h), and control (2.94 ± 0.43 h). There was no

difference between the preloads in the latency time (tlat) which represents the initial delay in the cumulative $^{13}C$ excretion curve. The ascension time (tasc), the time between the latency time and the half excretion time during the period of high $^{13}C$ excretion, was significantly different between all preloads ($p < 0.00001$). The longest times were after the soy and meat preloads, followed by the nuts and legumes preload, and then the dairy and control preloads. Similarly Rmax(out), the maximum rate of label excretion, was slowest after the soy preload ($0.19 \pm 0.06\ h^{-1}$), followed by the meat ($0.20 \pm 0.05\ h^{-1}$) and nut and legumes ($0.21 \pm 0.04\ h^{-1}$) preloads, and the dairy ($0.22 \pm 0.04\ h^{-1}$) and control ($0.23 \pm 0.04\ h^{-1}$) preloads had similar rates.

The self-corrected analogs representing the input to the bicarbonate pool were also analysed (Table 3.3.7). tlag(in) was fastest after the soy and meat preloads, followed by the nuts and legumes and dairy preloads, with the longest time after the control preload ($p = 0.0007$). There was no difference between the preloads for t1/2(in) ($p = 0.59$). The tlat(in) followed the same pattern as the tlag(in), with soy and meat preloads having the fastest latency input time and the control preload the slowest ($p = 0.003$). The tasc(in), as in tasc(out) was slowest after the soy and meat preload, then the nuts and legumes preload, and the dairy and control preloads were similar with the fastest times ($p < 0.0001$). There were no differences between preloads for Rmax(in), the maximum rate of label absorption ($p = 0.55$)

**Table 3.3.7 Gastric emptying self corrected parameters (mean ± sd)**

| Preload Meal | tlag in (h) | t1/2 in (h) | tlat in (h) | tasc in (h) | Rmax(in) ($h^{-1}$) |
|---|---|---|---|---|---|
| **Control** | 0.50 ± 0.18 | 1.14 ± 0.15 | 0.11 ± 0.06 | 0.28 ± 0.46 | 0.53 ± 0.07 |
| **Meat** | 0.30 ± 0.22 | 1.10 ± 0.19 | 0.05 ± 0.06 | 3.22 ± 0.68 | 0.56 ± 0.12 |
| **Dairy** | 0.39 ± 0.23 | 1.08 ± 0.22 | 0.07 ± 0.07 | 2.91 ± 0.46 | 0.57 ± 0.12 |
| **Nuts/ legumes** | 0.37 ± 0.22 | 1.10 ± 0.24 | 0.07 ± 0.57 | 3.03 ± 0.51 | 0.57 ± 0.19 |
| **Soy** | 0.28 ± 0.23 | 1.14 ± 0.19 | 0.05 ± 0.06 | 3.43 ± 0.69 | 0.54 ± 0.10 |
| **p value between preload meals** | $p < 0.001$ | $p = 0.6$ | $p = 0.003$ | $p < 0.001$ | $p = 0.5$ |

## 3.4 Discussion

In this study, four high-protein meals differing in protein type and a standard protein control meal were investigated for the short-term effects on the control of appetite, gastric emptying and post-prandial metabolism and subsequent energy intake in a classical preload design. The study was performed in a metabolic suite under standardised conditions to optimise investigation of the physiological effects of the meals.

The type of protein used in the study reflect typical dietary sources and were of topical interest given their epidemiological associations with cardiovascular and other health benefits, and the environmental advantage of consuming vegetable rather than animal protein. Soy is increasing in popularity in Western cultures because of benefits seen in relation to cardiovascular disease and bone density in Asian cultures with habitual high consumption, but little is known about the effect on energy intake. Nuts have been extensively studied for their cardiovascular benefits but concerns have been raised about the detrimental effect on energy intake and body mass of consuming such a high energy-dense food. Dairy products are of interest as recent concerns about their high saturated fat content have led to decreased consumption, which in turn may have led to inadequate calcium intake, yet dairy is claimed to aid weight loss. Meat was chosen for the standard protein and one of the high-protein preloads as the most common protein type habitually consumed which could therefore be a comparator to the other protein sources.

### 3.4.1 Effect of different protein types on satiety and energy intake

This study demonstrated that preload meals differing in protein type and quantity exert differential effects on satiety and subsequent energy intake. Energy intake after the soy high-protein preload was 11.7% lower than after the standard protein preload with a

difference of 538 kJ. This finding is reinforced by subjective assessments of hunger and satiety in which the soy preload produced lower hunger scores immediately after the meal, higher fullness scores prior to the test meal and reduced prospective consumption of food scores midway between the preload and test meal.

Protein has traditionally been said to be the most satiating macronutrient. With the exception of the soy preload, energy intake after the high-protein preloads was no different to the standard protein preload. This finding is similar to previous studies investigating short-term energy intake after meals differing in the proportion of protein (Stubbs *et al.*, 1996), (Raben *et al.*, 2003) despite a number of studies demonstrating a reduction in energy intake after protein-rich meals (Poppitt *et al.*, 1998), (Barkeling *et al.*, 1990), (Latner & Schwartz, 1999; Vozzo *et al.*, 2003) (Porrini *et al.*, 1997). The discrepancy in these data sets is possibly due to the proportion of energy from protein in the preload meals. With the exception of Vozzo *et al*, the proportion of energy from protein was between 43% and 72% in studies mentioned above which demonstrated enhanced satiety compared with other macronutrients. In comparison, the high-protein preload provided 31.8% of energy in a similarly designed study which did not demonstrate any difference in satiety or energy intake (Raben *et al.*, 2003). 33% of energy from protein used in high-protein preloads in this study may therefore have been too low to distinguish differences between the preloads in subsequent energy intake and satiety but reflects a more realistic intake. Initially the intention was to prepare meals containing a higher percentage of energy from protein. However, in order to balance the protein and fat content (to keep the fat content constant across all preload meals), and prepare the preloads with whole foods, a high rather than very high proportion of energy from protein was required in the meals. The high-fat content of the main protein source of the nuts and legumes meal was particularly limiting.

It is possible that the energy content of the preloads limited the effect on energy intake. However, at 2.5 MJ the meals were designed to be adequate to demonstrate an effect of the components of the meals. A preload of less than 1 MJ is insufficient to produce changes in energy intake (Poppitt *et al.*, 1998), whilst a saturating effect of larger meals (5 MJ) may exist (Lang *et al.*, 1998).

Although the preloads were designed with very similar ingredients and were visually very similar, the different protein types significantly affected palatability. The control preload, which had a composition close to habitual macronutrient composition, scored the highest for tastiness and was more enjoyable. Scores were significantly lower after the soy preload and the nuts and legumes preload for tastiness, pleasantness, and satisfaction and these preloads were less enjoyable. Palatability of the preload meal has been shown to affect energy intake at the test meal (Barkeling *et al.*, 1990) but energy intake was lower after the soy preload only and not after the nuts and legumes preload. These two preloads contained ingredients that the study population may have rarely eaten and so the unfamiliarity could have reduced their palatability.

An effect of soy on short-term energy intake has not been identified previously, however, a reduction in energy intake is consistent with data from a six-month intervention in which 90 subjects were provided with an energy restricted diet with or without additional soy protein supplements, and those provided with the soy supplements showed greater weight loss (8.9 kg) than the standard diet (6.2 kg) (Deibert *et al.*, 2004). While the effect size in the present study is small in the context of a specific hypoenergetic diet for the treatment of established obesity, it may be an important component of the overall diet for the prevention of weight gain. Hill *et al* (2003) suggested that decreasing energy intake

by approximately 420 kJ (100 kcal) per day is potentially useful for population-based weight management (Hill *et al.*, 2003).

Energy intake after the nuts and legumes preload was 8.8% higher than after the control preload. This finding was surprising and is in contrast to longer-term studies where nuts consumed in addition to a standard diet did not increase total energy intake (Hollis & Mattes, 2007) or change in weight (Fraser *et al.*, 2002a) (Sabate, 2003). More subjects were unable to finish the nuts and legumes preload than the other preloads, suggesting an immediate effect of increasing satiation. In keeping with this finding, the hunger scores after the nuts and legumes preload fell immediately after eating and were lower at 90 min than after the other preloads, and lower scores for prospective food consumption were also present. However, the resulting reduction in energy intake at the preload meal may have increased later energy consumption. When the total energy intake for the investigation day (preload and test meals) was calculated, excess consumption for the nuts and legumes investigation day was lower than the excess energy calculated at the test meal alone. Interpreting the impact of this diet is complicated since, because of difficulties in formulating this preload while maintaining the correct macronutrient proportions, the nuts and legumes preload was also higher in fibre (Table 3.2.2) and lower in glycaemic index (Figure 3.3.18) than the other high protein preloads. As high fibre, low glycaemic index meals are reported to increase satiety and energy intake (Hulshof *et al.*, 1993) (Roberts, 2000), it was therefore unexpected that the energy intake at the test meal after this preload was the highest.

Bowen *et al* (2006c) introduced an appetite score which combines the four questions relating to hunger, fullness, desire to eat and prospective consumption of food (Bowen *et*

*al.*, 2006c). It might be expected that the integrated assessment would provide a more rounded approach but there were no differences in the scores after the preload meals.

Surprisingly, scores for contentment and alertness were lower after both the soy and nuts and legumes preload. The lower immediate contentment scores may have been due to the lower palatability scores but it is not clear why lower scores were obtained for alertness prior to the test meal after these two preload meals. It is unlikely that these low scores affected the energy intake at the test meals as energy intake was reduced after the soy but increased after the nuts and legumes preload. The meat preload produced higher scores for contentment, lower scores for irritability, and a higher score for depression. The meat preload was the preload most similar to a common UK recipe and the familiarity and increased palatability may have enhanced contentment and reduced irritability. The highest maximum score for depression after the meat preload is not consistent with these results and is unexplained. These questions were primarily added to the questionnaire to reduce the focus on appetite and allow for a covert analysis of satiety. It was not expected that significant differences would be found between the investigation days. The differences are however important as factors such as contentment after a meal, alertness, and irritability determine whether an alteration in meal composition is acceptable and may thus be included in habitual eating patterns.

### 3.4.2 Mechanisms for the effect of protein type on satiety, energy intake and metabolic risk factors

A number of mechanisms for the effect on satiety and energy intake after preload meals differing in protein type were investigated in this study. Consistent with the finding of increased satiety and reduced energy intake after the soy preload, gastric emptying was

slower after the soy preload than the other preloads. Delayed gastric emptying has been associated with increased satiety and reduced energy intake (Hall *et al.*, 2003; Bowen *et al.*, 2006c) and also with a reduction in post-prandial glycaemia (Ma *et al.*, 2009). High-protein meals have previously been shown to have slower gastric emptying than high-carbohydrate meals (Ma *et al.*, 2009) but the effect of protein type on gastric emptying has not been previously reported. A differential effect of protein type on gastric emptying, may therefore account for some of the differences in satiety and energy intake that were observed in this study.

Marked differences in post-prandial glucose and insulin concentrations were observed in this group of healthy, lean subjects despite similar carbohydrate content of the high protein preload meals. It appears that protein is a powerful modulator of glucose metabolism, particularly when the meal contains non-animal protein (nuts, legumes and soy) perhaps due to their higher fibre content or lower GI. As large fluxes in post-prandial glucose and insulin may increase hunger, particularly when the glucose level falls below baseline prior to the subsequent meal, this effect on glucose metabolism may be an important mechanism by which different protein types influence satiety. The effect on satiety of reducing glucose excursions has been investigated with carbohydrate meals differing in their glycaemic index (GI) - the area under the glycaemic response curve during a 2-hour period after consumption of 50 g carbohydrate from a test food (where values are expressed relative to the effect of either white bread or glucose). When meals of similar carbohydrate content but differing in GI are provided, a low GI meal results in lower glucose excursions and is associated with a reduction in post prandial satiety (Arumugam *et al.*, 2008) and an increase in energy intake at a subsequent meal (Roberts, 2000). Similarly the effect of different protein types on post-prandial glucose excursions may influence post-prandial satiety and subsequent energy intake.

It is possible that the nature of the carbohydrate in the high protein preload meals in this study may have influenced post-prandial glucose metabolism, despite similar carbohydrate content. All the meals contained potato flakes, a high GI form of potato, however the nuts and legumes and soy preloads had very little potato in comparison to the other high protein preloads and the control preload. Carbohydrate in these two preload meals was comprised of ingredients substantially lower in GI – lentils in the nuts and legumes meal and soy mince in the soy meal – which may have lowered post-prandial glucose excursions. Additionally, fibre was higher in the nuts and legumes meal, further contributing to a reduction in glucose secretion.

The post-prandial peak concentrations of glucose and insulin were lowest after the nuts and legumes preload and the least post-prandial glucose dip was observed compared to the other preloads. This time period coincided with the lowest scores for prospective consumption of food, suggesting the larger post-prandial dip increased hunger. If food had been available between the two meals, the lower post-prandial blood glucose dip after the control and dairy preload may have contributed to increased food consumption. Glucose and insulin concentrations were similar prior to the test meal, suggesting that the influence on energy intake at this meal was not as pronounced.

An effect on post-prandial glucose and insulin levels of different protein meals may also provide metabolic benefits, and a reduction in cardiovascular risk is seen when glucose excursions are low (as discussed in Chapter 1). Nut consumption is associated with a reduction in cardiovascular disease (Hu *et al.*, 1998; Albert *et al.*, 2002) and developing diabetes (Jiang *et al.*, 2002) which may in part be explained by a reduction in post-prandial glucose concentration. A similar effect of nuts on glycaemic excursions was

seen in 15 subjects who consumed meals based on rice, almonds or potatoes of the same macronutrient composition. Glucose and insulin concentrations were lowest after the rice and almond meals than the potato meal and, additionally, lower oxidative damage to proteins was observed, suggesting that cardiovascular disease risk may be reduced by lowering post-prandial glucose and thus oxidative damage (Jenkins *et al.*, 2006).

Therefore, despite similar total carbohydrate content of the preload meals differences in protein types and associated changes in the nature of carbohydrates may exert a differential effect on glucose metabolism, which leads to changes in satiety and subsequent energy intake and may account for observed improvements in metabolic risk with certain protein types.

The post-prandial NEFA concentrations were similar after all meals except for the nuts and legumes meal. Higher levels of blood glucose and insulin levels promote the incorporation of free fatty acids into adipose tissue triacylglycerol so the delayed fall in NEFA may have been due to attenuation of the glucose increment after the nuts and legumes meal.

### 3.4.3 Strengths and limitations

This highly controlled crossover study conducted under standardised conditions allowed for consistent within-subject comparisons to investigate the physiological effects of the controlled preload meals of differing composition. Subjects were not aware of what was being investigated or in what ways the meals differed so this covert design reduced conscious alterations in eating behaviour.

The preloads were well matched for macronutrient composition and energy intake, and used standard household ingredients, provided in a recognisable form similar to a standard meal. A control preload with protein composition similar to the habitual diet was provided to compare with the high-protein preloads, in contrast to many preload studies which lack a control. The proportion of energy from protein in the high-protein preloads was designed to be only slightly higher than recommended intake and whole foods were selected in order to make the meal manipulation sustainable long-term and capable of being translated into real life despite the experiments being performed within the confines of a metabolic suite. The preload meals were designed to be relatively low in fat (lower than standard UK intake) and fat was kept constant due to the large effect of fat on energy intake and satiety (Stubbs *et al.*, 1995a). The variability in the type of carbohydrate between the control and the high-protein preloads did potentially affect the post-prandial glucose and insulin response but reflects the full consequence of manipulating the source of protein in the diet.

Meals were provided at times close to standard meal times in order to replicate habitual eating patterns and therefore extrapolate the results as far as possible to a free-living environment. An alternative method to investigate satiety is to record the time until subjects request a subsequent meal after a preload meal. A preload with higher satiating properties delays the time until the next meal, but consumption at the subsequent meal may not be affected. The objective of the present study was to assess energy intake at a second meal and so a set mealtime was selected.

The test meal provided choice of quantity of food with one savoury and one sweet dish. The nutritional composition of each dish was similar to that of the control meal to resemble habitual food consumption, and the meals were designed so that each mouthful

would contain the same ingredients and composition. The pizza and yoghurt dishes were liked by all participants, and had high palatability ratings. Subjects' consumption was determined by appetite as the pizza was provided in small slices which impeded calculation of their intake as a proportion of the whole, pre-empting habitual pizza eating – eating a half or whole pizza. Subjects were able to serve their own portion of yoghurt from the large serving dish and to eat to satiety.

The main limitation of the preloads was that visual appearance differed slightly. Despite the basic ingredients being the same in all preloads the different protein type altered the appearance, size, texture and palatability of the preloads. When the preloads were designed, it was decided to prioritise the macronutrient composition and the use of standard ingredients with the consequence that the fibre content, energy density and fatty acid profile differed in meals containing the different protein types. An attempt was made to balance the high levels of monounsaturated fatty acids in nuts triacylglycerols by adding olive oil to preloads if any extra fat was required and therefore reduce the differences in fatty acid profile.

The preload meal was 2.5 MJ, which was lower than originally intended, comprising less than one third of habitual daily intake. However pilot testing of the preload meals suggested that larger meals might have been difficult to consume in entirety and indeed not all subjects could complete even the 2.5 MJ meal. As the preload was baked and contained ingredients more commonly eaten in the evening rather than after an overnight fast, some subjects found the unfamiliarity made consumption of the meal difficult. There is a suggestion that a larger size meal is necessary to produce full effects on satiation (de Graaf *et al.*, 1992) and therefore the energy content of the preload meal may have been too low to observe the full satiating properties.

Visual analogue scales are a well-validated method for analysing satiety sensations (as discussed in Chapter 2) however there are inherent problems in any such questionnaire. Because of the frequency and repetitiveness of the questions subjects may not read the question correctly or think carefully about their response. Some subjects provided low scores for both fullness and hunger or provided an inconsistent pattern of scores throughout the investigation day, which may have affected the satiety results.

### 3.4.4 Summary

After consuming a high-protein soy-based preload, subjects experienced a significant suppression of energy intake compared to a standard meal of lower protein. An associated increased satiety was also observed, mediated in part by delayed gastric emptying and reduced post-prandial glucose and insulin excursions. Moreover there was a significant reduction in energy intake following high soy protein relative to meals of similar absolute protein content but from meat, dairy, nut and legume sources. If the effect on energy intake is sustained beyond a single meal, consuming meals rich in soy protein may improve body-mass control.

High-protein meals are often associated with increased satiety and energy intake compared to a meal of standard protein composition. An effect on these factors was not clearly seen in this study, possibly due to meal size or that the 33% of energy provided by the high protein meals was not sufficient to maximally influence energy intake.

A similar increase in satiety was observed after a high-protein preload containing nuts and legumes, but it did not translate to a reduction in energy intake during *ad libitum*

eating after the preload. However, the nuts and legumes meal appeared to be associated with increased meal satiation, an important factor in energy regulation in usual eating patterns, where meal size and timing are unrestricted. Additionally, the lower glucose and insulin excursions after the nuts and legumes preload have the potential to improve metabolic risk factors and cardiovascular disease.

This study highlights the differing effect of protein types on energy intake, satiety, gastric emptying and glucose and insulin concentrations after a meal, which may in part account for the discrepancies in previous studies of high protein meals reporting satiety and energy intake.

# 4 Chapter 4 Measuring the effects on energy metabolism of isoenergetic manipulation of the protein:non-protein energy ratio under *ad libitum*-feeding conditions.

## 4.1 Introduction

Evidence presented in Chapter 1 (Section 1.5) suggests that diets with a high-protein:non-protein energy ratio (P:E) may facilitate weight loss and enhance weight maintenance. Short-term experimental studies presented in Chapter 3 show that dietary protein can reduce immediate and subsequent food intake by early termination of eating, delaying eating and/or reducing intake at the next meal, suggesting that the action of protein on satiety may be a critical factor in the control of energy intake.

Evidence from longer well-controlled studies is limited. Westerterp-Plantenga *et al* (1999) demonstrated enhanced medium-term satiety in 8 subjects confined in a calorimeter, consuming an isoenergetic diet containing 29% or 9% of energy from protein at normal meal times over 24 hours (Westerterp-Plantenga *et al.*, 1999). Reported satiety was higher during meals and over the 24 h period among subjects eating the high-protein diet compared to the low-protein diet, indicating that in a highly controlled setting with limited food choice and availability, increasing the proportion of energy from protein increases satiety, and the effect is maintained for many hours. In a subsequent study, 12 subjects ate an isoenergetic diet comprising 30% or 10% of energy from protein for 4 days. On the fourth day, while confined to a calorimeter, satiety scores among subjects on the high-

protein diet were higher and hunger scores lower before and after dinner and for the whole 24 h period (calculated by AUC) (Lejeune *et al.*, 2006). These studies, performed in highly controlled settings with fixed food intake limits the translation of these observations to a free-choice setting.

In a detailed longer-term study, 19 subjects were placed on a weight-maintenance diet (15% P, 35% F, and 50% C) for 2 weeks, followed by an isoenergetic high-protein diet (30% P, 20% F, and 50% C) for 2 weeks, then an *ad libitum* high-protein diet (30% P, 20% F, and 50% C) for 12 weeks (Weigle *et al.*, 2005). During the isoenergetic high-protein diet there was no change in weight but visual analogue scores of hunger were reduced and those of fullness were increased. When the high-protein diet was consumed *ad libitum*, subjects reduced their energy intake by 1.8 MJ/day and experienced an average decrease in body mass of 4.9 kg over the 12-week period. Despite the lack of a control group in this study, the enhanced satiety and reduced energy intake observed demonstrated a substantial effect of the high-protein diet.

### 4.1.1 Protein and energy intake: The protein leverage hypothesis

There is a substantial body of evidence that protein intake in animals is tightly regulated to reach a specific 'intake target'. Experimental evidence comes from a range of herbivorous and carnivorous animals, including caterpillars (Lee *et al.*, 2002), grasshoppers (Raubenheimer & Simpson, 2003), carnivorous ground beetles and spiders (Mayntz *et al.*, 2005), rats (Simpson & Raubenheimer, 1997) and mice (Sorensen *et al.*, 2008). This intake target is chosen to maximise growth and reproductive capacity (Simpson & Raubenheimer, 1997). Simpson and Raubenheimer (2005) have developed the 'protein leverage hypothesis' which describes the prioritisation of protein over carbohydrate and

fat, such that protein intake determines total energy intake (Simpson & Raubenheimer, 2005). Animals select food that meets their nutrient requirements (the nutrient target) whilst providing for optimal growth or maintenance of body composition (the growth target). The ratio of protein to non-protein food that the animal eats in ideal conditions is termed the 'intake target' (Figure 4.1.1 (a)). When food availability is limited, animals prioritise absolute protein intake. If the proportion of protein in the food supply is limited animals over-eat carbohydrate and fat to ensure adequate protein is ingested (Figure 4.1.1 (b)). If available food is high in protein, animals become satiated after consuming a sufficient amount of protein, resulting in under-eating carbohydrate and fat, and hence energy. Accordingly, small changes in the percentage of dietary protein can sunstantially effect total energy intake, as the absolute amount of protein is prioritised while carbohydrate and fat, the major energy providing nutrients, are secondary (Simpson & Raubenheimer, 1997).

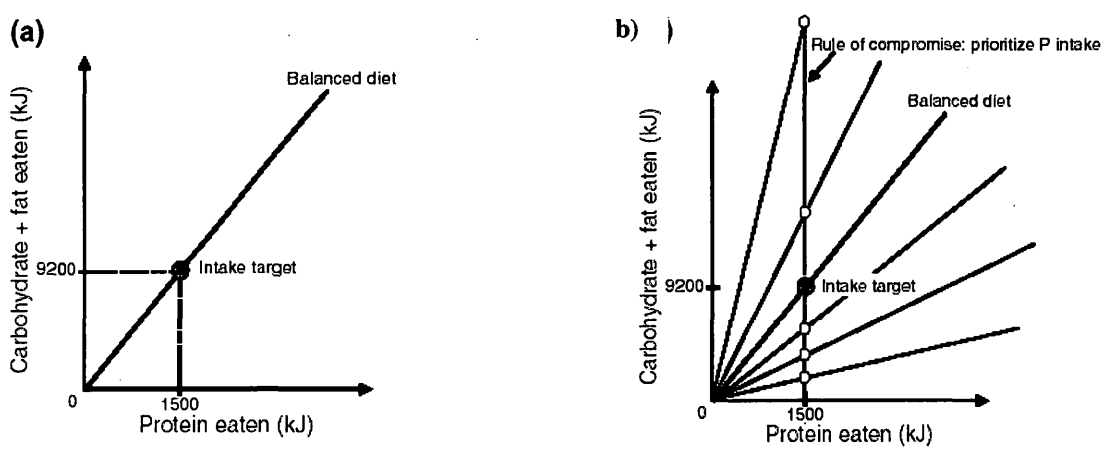
It is uncertain whether a particular macronutrient drives a similarly regulated system in humans. If protein intake modulates total energy (and therefore carbohydrate and fat) intake, changes in food availability or meal patterns which alter protein intake, may influence total energy intake and thus provide a mechanism for long-term body mass regulation. If protein intake is diluted by carbohydrate and fat (as may occur when these nutrients are more affordable or accessible), carbohydrate and fat may be over-consumed to maintain absolute protein intake leading to an increase in total energy consumption.

Experiments reported by Stock, 1999, support the hypothesis that protein is important in regulating energy balance and investigated energy balance at different levels of protein intake. A diet comprising 20% of energy from protein produced the highest efficiency of energy utilisation. When the proportion of energy from protein in the diet was reduced to

as low as 5% dietary induced thermogenesis increased. It was argued that this increase in heat production occurred in response to the need to increase consumption of fat and carbohydrate in response to the nutrient deficient low-protein diet, and was termed homeostatic waste. Likewise, an increase in protein increased DIT, due to the high metabolic cost of metabolising protein (Stock, 1999). For the purposes of this study the 10%P, 15%P and 25%P diets were defined as low, medium and high protein.

**Figure 4.1.1 Protein leverage hypothesis**

a) the P:E intake target in ideal eating conditions and b) the change in carbohydrate and fat intake required when food availability alters in order to maintain protein intake (Simpson and Raubenheimer, 2005).




As discussed in Chapter 1, energy intake and the onset of satiety in humans involve a complex system of sensory, cognitive and physiological responses to the presence of food. Regulation of this system is less likely to be driven by a single factor, such as dietary protein, as appears to occur in most animals studied to date. The increasing rates of overweight and obesity suggest that humans are not able to readily select their nutrient or growth targets. Archaeological evidence suggests that hunter-gatherer societies, who consumed a diet high in protein, were leaner and taller than subsequent societies who consumed grain-based diets (O'Keefe & Cordain, 2004). It is possible that hunter-gatherer

populations may have had more physiologically regulated food consumption. As the available protein was high, individuals could eat to their intake target while maintaining optimal body mass. As food choice and availability increase, particularly food with added fat and sugar (such as sugar-sweetened beverages which now constitute 16% of total energy in the US (Malik *et al.*, 2006)), the relationship between food consumption and physiological responses may lessen, resulting in weight gain and obesity. If, however, increased protein is available, humans may respond by eating to their appropriate intake target and reduce weight gain.

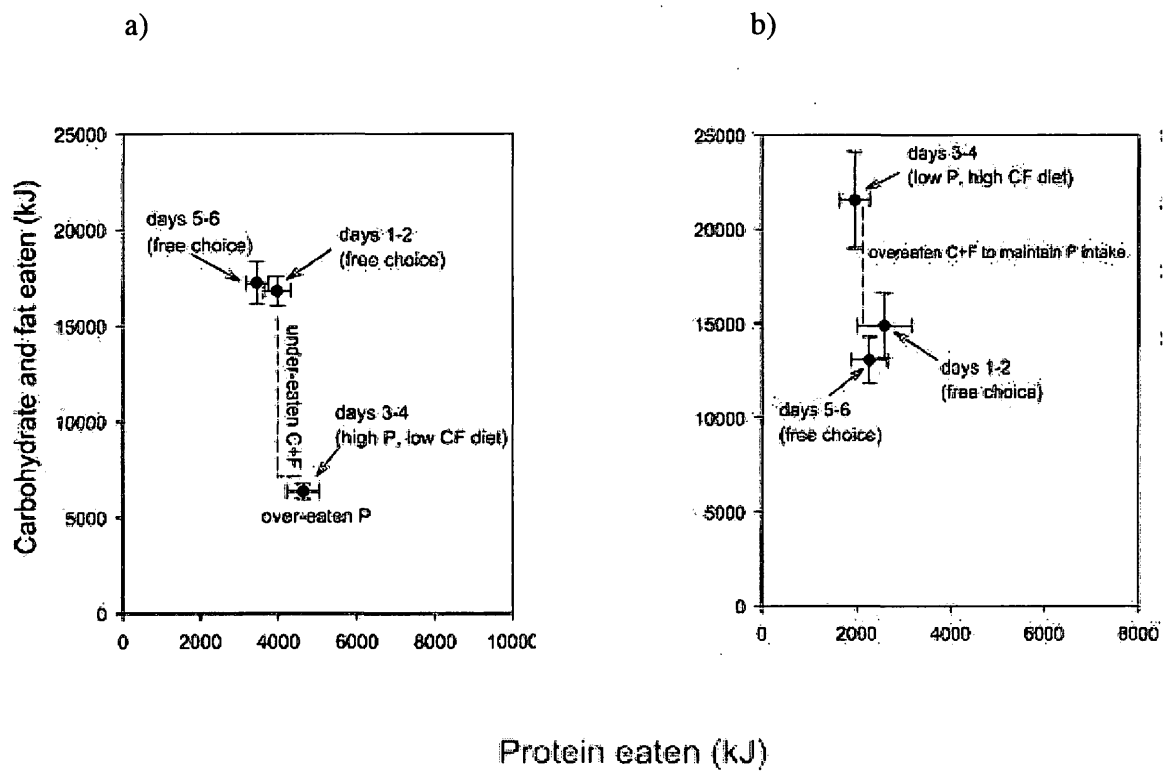
One preliminary study tested the protein leverage hypothesis in humans (Simpson *et al.*, 2003). Five female and five male white, health-conscious subjects aged 14 – 49 y were provided with all meals during a six-day residential experiment. Food selected from a buffet was consumed *ad libitum*, with a wide range of choice on days 1 and 2, and days 5 and 6. On days 3 and 4, half of the subjects were provided with a variety of food high in protein, while the other half were provided with food low in protein. The results are presented in Figure 4.1.2.

When food high in protein was offered, subjects increased their protein intake by 16% and reduced their carbohydrate and fat intake by 62%. Total energy intake fell by 47%. On returning to a habitual diet protein intake was reduced, suggesting a compensatory response to the protein excess consumed over the previous 2 days (Figure 4.1.2a)). Subjects offered food low in protein on days 3 and 4 reduced protein intake by 20% and increased carbohydrate and fat intake by 45%. Total energy intake increased by 35%. There was no compensatory increase in protein intake or reduction in carbohydrate and fat intake on days 5 and 6 relative to consumption on days 3 and 4 (Figure 4.1.2(b)). Subjects appeared to regulate protein intake over and above carbohydrate and fat, and adjusted

their total energy intake to maintain an absolute intake of protein. Therefore, protein may exert similar regulatory control over intake in humans as in wild and laboratory animals.

**Figure 4.1.2 Mean (SEM) intake over each of the 2-day phases of the experiment to test the protein leverage hypothesis in humans.**

(a) under-consumption of carbohydrate (C) and fat (F) for subjects offered food high in protein and (b) over-consumption of C and F for subjects offered food low in protein on days 3 and 4. (Simpson *et al*, 2003)




There were a number of limitations to this study. The study was a parallel design with few subjects not representative of the general population, and there was large inter-individual variability in food intake. Food provided on days 3 and 4 was not controlled for a number of factors known to affect satiety and energy intake (as discussed in Chapter 1) including palatability, energy density, fibre, variety and visual appearance. As subjects were only provided with one of the two protein-manipulated menus, the outcomes could not be compared within-subject, and food choices may have been affected by the

communal nature of food selection and consumption. Further testing is required with a more controlled dietary manipulation with a within-subject design.

### 4.1.2 Dietary protein and metabolic risk

#### *4.1.2.1 Body composition*

Evidence suggests that diets with higher P:E ratio may promote weight loss, improve body composition and reduce fat deposition in adipose tissue, alter lipid metabolism, lower post-prandial blood glucose, enhance insulin sensitivity, and alter growth factors and hormones involved in glucose metabolism.

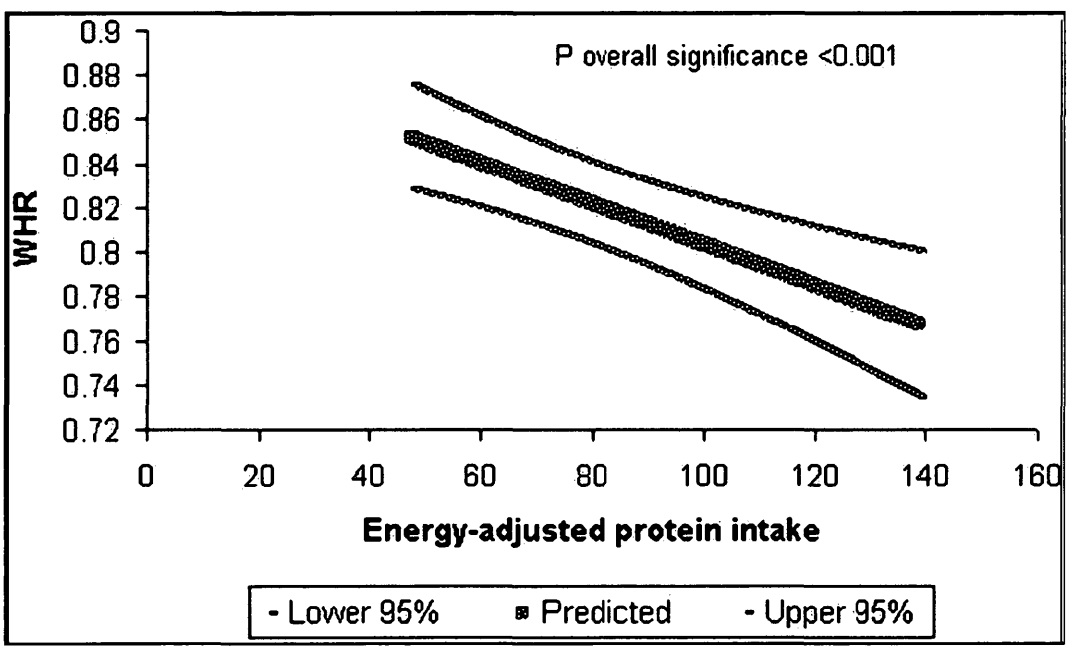
Fat mass, particularly abdominal fat, is a key component in the diagnosis of the metabolic syndrome and is associated with an increased risk of cardiovascular disease (Eckel *et al.*, 2005). Therefore reducing fat mass or improving the ratio of lean to fat mass is expected to have metabolic benefits. For example, in mice susceptible to weight gain, increasing the protein content of the diet over 10 weeks delayed weight gain and reduced the quantity of fat gained, from less than 1 g in the high-protein/low-fat diet, to 5 g in the low-protein/low-fat diet, to almost 8 g in the high-protein/high-fat diet ($p < 0.05$) (Klaus, 2005).

In 617 adults reporting habitual high consumption of protein, in food frequency questionnaires, substituting protein for carbohydrate (but not fat) resulted in a lower WHR by 0.0005 for every gram per day increase in protein intake (Figure 4.1.3) (Merchant *et al.*, 2005). Similarly, among 20,126 women there was an inverse relationship between protein intake and the change in waist circumference over five

years. Interestingly no association between total energy or energy from fat and waist circumference was observed (Halkjaer *et al.*, 2006).

**Figure 4.1.3 The relationship between WHR and protein intake (g/d)**

After multivariate adjustment in study participants (*P* for significance of overall spline < 0.001). Adjustments were made for age (y), total energy (kJ/d), height (cm), physical activity score (continuous variables), sex (dichotomous), BMI (kg/m2) (continuous), smoking (never, past, current), alcohol intake (never or <1 time/mo, 1 time/mo to 5 times/wk, >5 times/wk), and ethnicity (Aboriginal, South Asian, Chinese, European). (Merchant *et al.*, 2005)




During energy restriction, body mass is lost from both fat stores and from lean body mass. However, if whilst reducing body mass, partitioning of energy stores preserves lean mass, a higher proportion of fat tissue is lost. There is evidence to suggest that increasing the proportion of protein in the diet can maintain lean tissue during weight loss and thereby increase the amount of fat lost.

Partitioning of energy stores was demonstrated in 48 overweight women, consuming an energy-restricted diet high in protein or carbohydrate over four months, in whom body mass was reduced by 9.3 kg (7.3 kg fat mass, 2 kg lean mass) in the high-protein group (30% of energy from protein) with relative preservation of lean mass compared to 7.6 kg body mass loss ($p < 0.05$) (5.0 kg fat mass, $p < 0.05$, 2.6 kg lean mass, $p = 0.1$) in the high-carbohydrate group (15% of energy from protein) (Layman *et al.*, 2005).

Similarly, overweight women provided with a 10-week energy restricted diet, comprising 30% or 16% of energy from protein, lost body mass (7.5 kg high-protein, 7.0 kg low-protein) comprising significantly more fat in the high-protein group than the lower-protein group (5.6 vs. 4.7 kg; $p<0.05$) and sparing of lean tissue ($p <0.05$). (Layman *et al.*, 2003).

During *ad libitum* eating lean mass preservation was demonstrated in 148 subjects who consumed a weight-maintenance diet comprising 18% or 15% of energy from protein for three months, preceded by a four-week period of weight loss following a very low-energy diet (an additional 48.2 g/day of protein was consumed in the higher protein group) (Westerterp-Plantenga *et al.*, 2004). After 6.4 kg of weight loss, weight regained over three months was 1 kg of lean mass in the additional protein group compared to 2 kg of lean and fat mass in the control group. It is notable that this study demonstrated a beneficial effect on both weight regain and body composition despite only a small increase in protein consumption over the control diet. It is possible that subjects in the additional protein group were consuming a diet closer to their intake target, as described in the protein leverage hypothesis, and so were not required to overeat carbohydrate and fat to reach optimal protein intake.

In a further study during an *ad libitum* 30% protein diet 19 lean subjects experienced a 4.9 kg weight loss after 12 weeks, 76% of which was fat mass (Weigle *et al.*, 2005). No control group was included, however this study illustrates that weight loss occurring during *ad libitum* consumption of a comparatively high-protein diet results in an improved lean-to-fat mass ratio.

Altering the ratio of lean to adipose tissue during weight loss or weight maintenance has significant long-term health benefits. The data from intervention studies during energy restriction or weight maintenance suggests that a positive protein balance produces a beneficial change in body composition and may be one mechanism by which protein modulates metabolic risk. However, a direct relationship between a positive protein balance leading to a change in body composition remains to be demonstrated.

#### *4.1.2.2 Glucose metabolism*

Abnormalities in glucose metabolism and insulin sensitivity are an important component of metabolic risk. Impaired glucose metabolism may be the first indicator of risk of more serious disease such as type 2 diabetes, and is associated with an increased risk for cardiovascular disease. Fluctuations in insulin levels and rebound hypoglycaemia following elevations in glucose also appear to stimulate hunger (Bray *et al.*, 1998) leading to effects on satiety and weight regulation.

The post-prandial rise in glucose and insulin were lower after a protein-rich meal in 14 lean healthy subjects provided with five meals of different macronutrient composition (Erdmann *et al.*, 2004). Glucose decreased from 4.6 mmol/l to 4.2 mmol/l over 4 hours after a meal containing 86% of energy from protein, indicating significantly more

efficient glucose clearance than after high-fat or high-carbohydrate meals. Protein intake was considerably higher than is usual in habitual diets, so translating these data to the general population is problematic.

A more realistic study used a randomised crossover design in 23 overweight, otherwise healthy women. Over nine hours subjects ate snacks high in protein (morning snack 21.9% P, 19.4% F, 58.7% C, afternoon snack 39.7% P, 24.1% F, 36.2% C) or low in protein (<0.1% P, 35.5% F, 64.4% C). Glucose and insulin levels were lower when the high-protein snacks were eaten, particularly at 1 and 2-hour timepoints after the consumption of the snack bars (Williams *et al.*, 2006). Energy intake at an *ad libitum* buffet lunch and throughout the day did not differ between groups presumably because manipulating the composition of only the snacks had a modest effect on daily macronutrient intake.

The short-term reduction in post-prandial glucose may be maintained when a high-protein diet is adopted over several weeks. Eight men with mild untreated type 2 diabetes were randomised to five weeks of a high (30% P, 50% F, 20% C) or standard protein (15% P, 30% F, 55% C) diet in a controlled crossover design, with a five-week washout between diets (Gannon & Nuttall, 2004). Mean fasting blood glucose concentration reduced from 9.3 mmol/L to 6.6 mmol/L during the high-protein diet, while fasting glucose concentration did not significantly reduce in the standard protein diet (10 mmol/L to 8.8 mmol/L). Mean glucose concentration over 24 h and the 24 h response curves for glucose and insulin were all significantly lower during the high-protein diet. Glycohaemoglobin was reduced from 9.8% to 7.6% during the high protein diet but remained unchanged during the standard protein diet. As these subjects had type 2 diabetes the potential for improvement was greater than for healthy subjects, however the

reductions in glucose and glycohaemoglobin are remarkable over the short period studied.

### 4.1.3 Objective

The primary objective of this study was to determine the effect of dietary protein on energy intake by studying *ad libitum* energy intake on diets of varying P:E ratios (where protein provided 10%, 15% or 25% of energy) under standardised conditions, using a within-subject design. The secondary objective was to investigate the effect of changes in P:E ratio on body composition and various appetite control mechanisms, including circulating metabolites and appetite hormones.

## 4.2 Methods

This study was performed at MRC Human Nutrition Research (HNR) and the Wellcome Trust Clinical Research Facility (CRF) between March 2007 and December 2007. The study was approved by Cambridge Local Research Ethics Committee in November 2006 (Ref No. 06/Q0108/181), by the Addenbrooke's NHS Foundation Trust Research and Development Committee in January 2007 (Ref: A090877), and the Scientific Advisory Board for the Wellcome Trust Clinical Research Facility at Addenbrooke's Hospital in November 2006.

#### *4.2.1.1 Subjects*

##### 4.2.1.1.1 Sample size

There were no pre-existing data for studies on manipulation of the P:E ratio to estimate the sample size. Estimates were therefore based on the work of Stubbs *et al.*, (1995a) where *ad libitum* food intake was measured on three occasions over 7 days in 6 men,

confined to a calorimeter (Stubbs *et al.*, 1995a). Protein was held constant (13% of energy) but the proportion of dietary fat was manipulated so that the diets contained 20%, 40%, or 60% fat with reciprocal changes in carbohydrate. Mean ± SEM energy intake on the different diets was: 20% fat - 9.03 ± 0.25 MJ/day; 40% fat - 10.22 ± 0.29 MJ/day; 60% fat - 12.36 ± 0.41 MJ/day.

To detect a difference in energy intake of 0.5MJ/day using a crossover design, with a maximum SE of 0.41MJ/day, for 90% power at 5% significance, a sample size of 10 was required. It was therefore planned to study 10 completing subjects with any dropouts or non-completers replaced.

#### 4.2.1.1.2 Recruitment

Subjects were recruited from the general population in a similar manner to the previous study (3.2.1.2). The HNR volunteer database was searched, and all potentially eligible subjects were sent a letter, approved by the ethics committee (see Appendix II.2.4), inviting them to participate in the study. An advertisement (see Appendix II.2.6) was placed on a university student email newsletter. Information sheets and advertisements were also placed in the volunteer suite lounge to alert subjects who had been involved in other research studies at HNR.

Interested individuals were contacted to discuss the study in more detail and complete a telephone-screening questionnaire (see Appendix II.2.3) to determine eligibility. Subjects were then invited to HNR for a screening visit (see 4.2.2.1).

The general practitioner (GP) of subjects eligible to participate in the study was informed (see Appendix II.2.5) and asked to contact the chief investigator if there was any medical reason that would prevent participation in the study. All clinically relevant results were reported back to the GP with the consent of the subject.

#### 4.2.1.1.3 Eligibility

Male and female subjects were eligible to participate in the study if they were aged 18-70 years, with a BMI less than 25 $kg/m^2$, were willing to eat all the study foods, were unrestrained eaters (scoring less than 20 on the EAT-26) and were able to live in the research facility for the duration of the study.

Subjects were excluded if they were pregnant or planning pregnancy, breastfeeding, had known chronic disease – diabetes, cardiovascular disease, renal disease, liver disease, or untreated thyroid disease, unstable or untreated elevated blood pressure or cholesterol, chronic inflammatory conditions, were taking medications that might interfere with glucose metabolism or appetite, allergy or intolerance to any of the intervention foods, vegetarian, had previous history of an eating disorder, were following a weight-reducing diet or recently experienced significant weight change.

Subjects were asked not to alter lifestyle habits for the duration of the study visits, to continue their usual diet and exercise regimen and to not start or stop taking any dietary supplements during the four weeks prior to or for the duration of the study. Premenopausal women were studied in the same phase of their menstrual cycle to minimise the potential effects of reproductive hormones on appetite control (Buffenstein

*et al.*, 1995). To standardise the schedule the study aimed to measure all subjects 4 weeks apart.

### 4.2.2 Study design

Subjects completed the preliminary telephone questionnaire and attended HNR for a screening day. Those eligible to participate attended for 3 five-day periods of dietary manipulation, in random order, with approximately four weeks between each visit (see Table 4.2.1 for details of each experiment week). For the first three days of the five-day residential period, participants were provided with *ad libitum* food of fixed P:E ratio (10%, 15% or 25% protein). The days were spent at HNR and the nights at the Wellcome Trust Clinical Research Facility (CRF), Addenbrooke's Hospital. The last 36 hours of each experiment week was spent in a room calorimeter at the CRF. This study is described in Chapter 5. Transport was provided to move between the two sites so that participants were continuously supervised.

Subjects were advised that they were participating in a study to investigate the effects of different foods on metabolism and body composition. They were unaware of the manner in which the food was manipulated or that energy intake was being measured.

#### *4.2.2.1 Screening day*

Potential participants were invited to HNR to discuss the study in detail. After a full explanation of all the requirements of the study, written consent was obtained (Appendix II.2.2). A medical history was taken and all medications and supplements used were recorded. Subjects were asked to record medications they used during the study period or any appointments with their GP. Measurements were taken of body mass, height, waist

circumference, and blood pressure (as described in Chapter 2). BMI was calculated to ensure subjects met eligibility criteria. The Eating Attitudes Test (EAT-26) (Garfinkel & Newman, 2001)) (Appendix II.2.8) was completed to identify symptoms of an eating disorder and as a basis for excluding restrained eaters. The maximum score to be eligible for inclusion was 20 points. Participants also completed the International Physical Activity Questionnaire to assess habitual exercise (Appendix II.2.9). This questionnaire is designed for 15-69 year olds to record activity that has occurred over the previous 7 days (Craig *et al.*, 2003).

Subjects were instructed on how to complete a 4-day food diary, including 2 weekdays and 2 weekend days. Food diaries were completed prior to each experiment week to provide an estimate of habitual food intake and to document any changes in the P:E ratio of the background diet over the course of the study.

A visit to inspect the facilities and calorimeters at the CRF was arranged. All procedures performed at HNR and the CRF were explained in detail to the subjects who were also provided with information leaflets for future reference. Dates for the study visits were arranged to ensure that subjects were studied in the same phase of their menstrual cycle, and were free of other social engagements during the study visits and immediately prior to their study weeks, to maintain standard eating and exercise behaviour during the study period. Subjects were asked to avoid strenuous activity and alcohol for the 24 hours prior to commencing the study.

**Table 4.2.1 Study schedule**

| | Screening | Experiment Week | | | | |
|---|---|---|---|---|---|---|
| Performed at CRF | | Day 1 (Mon) | Day 2 (Tue) | Day 3 (Wed) | Day 4 (Thurs) | Day 5 (Fri) |
| Protein manipulated *ad lib*.diet | | | | | | |
| Protein manipulated isoenergetic diet | | | | | | |
| Food diary 4 days | | X | | | | |
| 24 hour urine | | X | | | X | |
| Medical history/exam | X | | | | | |
| Eating attitudes test | X | | | | | |
| Physical activity questionnaire | X | | | | | |
| Basal Metabolic Rate | | X | | | | X |
| Fasting blood analyses | | X | | | | X |
| Post-prandial blood analyses | | | | | X | |
| Height | X | | | | | |
| Body mass | X | X | | | | X |
| Blood Pressure | X | X | | | | X |
| Waist | X | X | | | | X |
| Whole Body DXA | | X | | | | X |
| Bod Pod | | X | | | | X |
| Visual Analogue Scale - Appetite | | | | hourly | | |
| Visual Analogue Scale - Palatability | | | | Post-prandial | | |
| Continuous glucose sensor | | | | | | |
| Calorimeter | | | | | | |
| Afternoon walk or exercise in calorimeter | | X | X | X | X | |

### *4.2.2.2 Experiment Week*

Day 0: Each subject arrived at the CRF at 6pm with their 4-day food diary. Subjects were admitted to the CRF and asked about recent illnesses or medication use that might prevent them from participating in a study visit. Subjects were provided with a standard meal providing 6.4 MJ (see Appendix II.1 for details) and asked to eat to satiety. They were then requested to fast from midnight.

Day 1: Basal metabolic rate (BMR) was measured before rising using a GEM indirect calorimeter (see 2.4.2 for details). Body mass, waist circumference, and blood pressure

were measured and a whole body DXA scan, and a Bod Pod Body Composition measurement was performed (as described in Chapter 2). Blood samples were taken for measuring fasting glucose, insulin, lipids, and hsCRP (section 4.2.3.4 and in 2.6.3).

Participants were transported to HNR once these investigations were completed. On arrival at HNR, subjects were advised that food was available a*d libitum* throughout the day but that no food from other sources could be consumed. Subjects were invited to request food at any stage and to consume sufficient food in order to be comfortably full after meals. All food was weighed before and any leftovers after eating. Meals were consumed in private booths and subjects were encouraged not to talk with other study participants whilst eating. Drinks were not limited but subjects were required to consume the same number of cups of tea or coffee each day. Subjects were provided with a flask containing 150 ml milk that could be used for hot drinks or added to the breakfast meal but had to be consumed within the day. A snack box was provided for consumption between meals whilst at HNR, and more snacks could be requested at any stage.

Participants spent an hour in the afternoon on a supervised walk. At other times they were confined to the metabolic suite. During this time, they had access to the internet, computers, television, reading material and desk space for craft activities, writing etc. After the evening meal, subjects were transported back to the CRF to spend the night. A snack box was provided for the evening which was removed at bedtime, or at midnight, sealed and returned to the investigators the next day.

Day 2: Participants were transported to HNR from the CRF after an overnight fast. Their snack boxes were emptied and remaining food weighed. Food and exercise was provided as day 1. A continuous glucose monitor was inserted in the afternoon (see 2.6.3.2).

Subjects were given full instructions about the care of the monitor and how to measure comparison capillary blood glucose levels. Subjects were given sheets to record the time of all activities, meals, exercise and blood sampling, and advised to measure blood samples and enter the results into the glucose monitor before every meal and before bed each day until the morning of day 5.

Day 3: Food and exercise was provided as for day 2. Visual analogue scales for the measurement of subjective hunger and satiety were completed hourly throughout the day. Visual analogue scales for palatability were completed after each meal. Subjects were transported to the CRF with weighed and prepared food for day 4, their evening snack boxes, and the remaining VAS questionnaires.

On returning to the CRF subjects entered the whole body calorimeter. An intravenous cannula was inserted before the calorimeter door was closed. They continued to complete hourly VAS until 2200 h and were able to consume food *ad libitum* from their snack boxes until bedtime.

Day 4: Participants spent the day in the calorimeter. The protocol is detailed in Chapter 5. Subjects started a 24-hour urine collection from the second urine sample of the day. Fasting blood samples were taken for glucose, insulin, lipids, and hsCRP. Basal and post-prandial measures of insulin, ghrelin, GLP-1, PYY, PP, IGF-I and IGF-BP3 were taken at 30-minute intervals after the first fixed-energy meal of the day until the second meal.

Day 5: Participants completed the 24-hour urine collection and then left the calorimeter. Repeat measures of basal metabolic rate, body mass, waist circumference, blood pressure, a whole body DXA scan, and air plethysmography measurements were

performed. The continuous glucose monitor was removed. Subjects were offered a free choice of breakfast and provided with transport to return home.

The same protocol was repeated for the two subsequent study visits.

#### *4.2.2.3 Dietary Manipulation*

All food provided was manipulated to comprise 10%, 15% or 25% of energy from protein (10%P, 15%P, 25%P) from mixed protein sources. Dietary fat was kept constant at 30% (in order to minimise changes in energy density) and the proportion of carbohydrate was adjusted to be 60, 55 or 45% of total energy. The 15%P diet was designed to be a control diet, most consistent with habitual diet in the UK population. The 10%P and 25%P diets were elected as low and high-protein diets respectively which were within the recommended minimum and maximum protein requirements, and to allow the preparation of diets that were palatable, able to be consumed over a long period of time, and which could be prepared using whole foods.

Each participant was studied on three occasions and on each occasion was provided with one of the three diets. The order of diets was randomised. There were six possible sequences for the order of diets and subjects were randomly allocated to one of these sequences (Table 4.2.2).

**Table 4.2.2 Diet sequences followed by subjects showing % energy from protein**

| Subject | Visit 1 | Visit 2 | Visit 3 |
|---|---|---|---|
| 1 | 25% | 10% | 15% |
| 2 | 25% | 15% | 10% |
| 3 | 10% | 15% | 25% |
| 4 | 15% | 10% | 25% |
| 5 | 15% | 10% | 25% |
| 6 | 15% | 25% | 10% |
| 7 | 25% | 10% | 15% |
| 8 | 10% | 15% | 25% |
| 9 | 15% | 25% | 10% |
| 10 | 10% | 25% | 15% |

The visual appearance of dishes for each macronutrient composition was matched as closely as possible to disguise the experimental manipulation of nutrient composition and all were informally pretested.

All meals were homogenous in nature to ensure that the dietary composition was correct irrespective of the total volume consumed. Each meal contained two different items – one savoury, one sweet - and the recipes were adjusted to produce food of the appropriate macronutrient composition. As described in Chapter 2 standard tables (FSA, 2002) were used to calculate the composition of meals. Fibre and energy density were kept constant as far as possible for each macronutrient composition of the same meal. A summary of

the composition of the meals is shown in Table 4.2.3 and Table 4.2.4. The meals are detailed in Appendix II.1.

The meals were prepared in the HNR volunteer suite kitchen following good food hygiene standards. Where appropriate, meals were frozen, then defrosted and heated by microwave oven prior to each investigation day to an internal temperature of 80°C according to a standard protocol.

All meals were offered in large serving dishes from which participants could select their own serving size, to ensure *ad libitum* intake. Snacks of the appropriate composition were provided in a snack box from which subjects could eat freely. All food provided and all leftover food was weighed to calculate energy intake. This methodology is comparable to previous highly controlled appetite studies (e.g. (Stubbs *et al.*, 1995a).

**Table 4.2.3 Macronutrient composition, fibre and energy density of the study meals per serving, days 1 and 3.**

| Meal and Diet type | Protein | Fat | Carbohydrate | Energy | Fibre | Energy Density |
|---|---|---|---|---|---|---|
| | % | % | % | kJ | g | kJ/g |
| **Breakfast** | | | | | | |
| **Bran flakes cereal** | | | | | | |
| 10%P | 10.2 | 29.7 | 60.1 | 3491 | 24.5 | 7.9 |
| 15%P | 14.8 | 30.1 | 55.1 | 3515 | 24.4 | 7.6 |
| 25%P | 24.6 | 30.3 | 45.1 | 3492 | 23.6 | 7.8 |
| **Lunch** | | | | | | |
| **Macaroni cheese** | | | | | | |
| 10%P | 10.4 | 30.0 | 59.7 | 5008 | 6.5 | 5.0 |
| 15%P | 15.1 | 30.1 | 54.8 | 4999 | 6.5 | 5.8 |
| 25%P | 25.0 | 30.1 | 44.9 | 5014 | 6.4 | 5.9 |
| **Raspberry yoghurt** | | | | | | |
| 10%P | 10.0 | 30.2 | 59.7 | 2496 | 8.0 | 5.4 |
| 15%P | 15.4 | 30.3 | 54.3 | 2504 | 8.9 | 4.3 |
| 25%P | 24.6 | 30.2 | 45.2 | 2502 | 9.1 | 3.3 |
| **Dinner** | | | | | | |
| **Mushroom chicken and rice** | | | | | | |
| 10%P | 9.9 | 30.0 | 60.1 | 5005 | 2.7 | 5.1 |
| 15%P | 15.2 | 30.0 | 54.9 | 5008 | 2.5 | 5.3 |
| 25%P | 25.0 | 29.7 | 45.3 | 4984 | 2.2 | 5.4 |
| **Bread and butter pudding** | | | | | | |
| 10%P | 10.0 | 30.1 | 60.0 | 2498 | 4.4 | 7.6 |
| 15%P | 14.9 | 30.3 | 54.7 | 2497 | 3.0 | 7.6 |
| 25%P | 24.7 | 30.3 | 45.1 | 2508 | 3.2 | 5.1 |
| **Snacks** | | | | | | |
| **Yoghurt muffins*** | | | | | | |
| 10%P | 10.0 | 29.9 | 60.1 | 2491 | 2.3 | 12.9 |
| 15%P | 15.1 | 30.3 | 54.6 | 2504 | 2.6 | 11.3 |
| 25%P | 25.1 | 30.7 | 44.3 | 2503 | 3.8 | 9.2 |
| **Cheese scones*** | | | | | | |
| 10%P | 10.1 | 29.9 | 60.1 | 5000 | 6.6 | 14.3 |
| 15%P | 15.1 | 29.9 | 55.0 | 4998 | 6.0 | 13.7 |
| 25%P | 25.0 | 30.0 | 45.0 | 5009 | 6.6 | 12.5 |
| **Tuna sandwiches*** | | | | | | |
| 10%P | 11.8 | 29.5 | 58.7 | 2554 | 3.6 | 11.3 |
| 15%P | 15.2 | 29.9 | 54.9 | 2504 | 3.3 | 10.9 |
| 25%P | 25.1 | 29.9 | 45.0 | 2501 | 10.5 | 9.5 |

**Table 4.2.4 Macronutrient composition, fibre and energy density of the study meals per serving, days 2 and 4**

| Meal and Diet type | Protein | Fat | Carbohydrate | Energy | Fibre | Energy Density |
|---|---|---|---|---|---|---|
| | % | % | % | kJ | g | kJ/g |
| **Breakfast** | | | | | | |
| **Porridge** | | | | | | |
| 10%P | 9.9 | 30.3 | 59.7 | 3508 | 13.9 | 3.7 |
| 15%P | 15.1 | 29.7 | 55.2 | 3499 | 13.6 | 3.7 |
| 25%P | 24.3 | 30.2 | 45.5 | 3511 | 12.6 | 4.7 |
| **Lunch** | | | | | | |
| **Vegetable and lentil curry** | | | | | | |
| 10%P | 10.3 | 30.0 | 59.7 | 5018 | 24.5 | 2.6 |
| 15%P | 15.2 | 30.0 | 54.9 | 5019 | 25.7 | 2.7 |
| 25%P | 24.8 | 29.7 | 45.4 | 4980 | 23.5 | 3.1 |
| **Strawberry yoghurt** | | | | | | |
| 10%P | 10.1 | 30.0 | 59.9 | 2508 | 6.2 | 5.7 |
| 15%P | 15.1 | 30.1 | 54.8 | 2517 | 7.1 | 4.6 |
| 25%P | 24.7 | 30.5 | 44.8 | 2515 | 6.3 | 3.5 |
| **Dinner** | | | | | | |
| **Bolognaise pasta** | | | | | | |
| 10%P | 10.8 | 29.9 | 59.2 | 5019 | 10.4 | 5.3 |
| 15%P | 15.0 | 29.9 | 55.1 | 4998 | 10.9 | 5.1 |
| 25%P | 25.1 | 29.7 | 45.2 | 4993 | 9.6 | 5.0 |
| **Rice pudding** | | | | | | |
| 10%P | 9.8 | 29.9 | 60.2 | 2504 | 0.4 | 5.2 |
| 15%P | 14.5 | 30.4 | 55.1 | 2500 | 0.4 | 5.7 |
| 25%P | 24.5 | 30.5 | 45.0 | 2509 | 0.3 | 4.4 |
| **Snacks (day 2)** | | | | | | |
| **Peanut butter sandwiches *** | | | | | | |
| 10%P | 11.5 | 30.4 | 58.1 | 2472 | 4.1 | 12.2 |
| 15%P | 15.4 | 29.6 | 55.0 | 2500 | 10.6 | 11.8 |
| 25%P | 17.8 | 42.6 | 39.6 | 2871 | 13.8 | 11.9 |

* Snacks were provided during the day and in the evening. The snacks on day 1 were yoghurt muffins and cheese scones during the day and in the evening, with the addition of raspberry yoghurt in the evening. On day 2 peanut butter sandwiches were provided during the day, and the evening snack was peanut butter sandwiches, yoghurt muffins, cheese scones and strawberry yoghurt. On day 3 the daytime snack was tuna sandwiches and the evening snack was tuna sandwiches, yoghurt muffins, cheese scones and raspberry yoghurt. No snack was provided on day 4.

### 4.2.3 Outcome measures

#### *4.2.3.1 Energy Intake*

Energy intake was calculated from the weight and composition of the food consumed.

#### *4.2.3.2 Palatability*

Visual analogue scales to assess palatability of the meal were completed after each meal and were completed separately for each course of the meal (Table 4.2.1).

#### *4.2.3.3 Hunger and Satiety scores*

Visual analogue scales to assess hunger, fullness, and mood were completed hourly on day 3 from 0900h to 2200h (Table 4.2.1). (See 2.3 for detail and Appendix I.2.7 for the questionnaire).

#### *4.2.3.4 Body composition*

Body composition was measured using Dual Energy X-ray absorptiometry (DXA) and air plethysmography (Bod Pod).

#### *4.2.3.5 Urine Samples*

24-hour urine collections were collected between days 4 and 5 of the experiment weeks and were measured as a crosscheck on protein intake during the dietary manipulation. Subjects' urine was measured and sampled from the time they entered the calorimeter until leaving the calorimeter 36 hours later. After each void, the urine volume was measured and 2 x 10 ml aliquots were collected in pre-labelled urine collection tubes

(Sarstedt, Leicester, UK). From the first void after 0700 h (the second void of the day) on day 4 of the study until the first void on day 5 of the study all urine was collected for a 24 h urine collection. The total volume was recorded and 2 x 25 ml aliquots were taken into a glass universal. Samples were stored in a refrigerator at –4 °C until analysis.

#### *4.2.3.6 Blood markers of metabolic risk and satiety*

10 ml blood samples were taken on days 1 and 4 of each of the three study visits. The blood samples were analysed for markers of cardiovascular risk: glucose, insulin, lipids, and high-sensitivity c-reactive protein (hsCRP).

On day 4, 11 ml blood samples were taken at -1, 30, 60, 90, 120, 150, 180, 210 minutes after eating the meal to measure post-prandial levels of glucose, insulin, ghrelin, GLP-1, PYY, PP, IGF-I and IGF-BP3 (described in 2.6.3).

Interstitial glucose measurements were recorded every 20 minutes from the evening of day 2 until the morning of day 5 using continuous glucose monitoring (2.6.3.2). The CGMS was worn for a sufficient duration to provide data from two full 24-hour (0700 h – 0700 h) periods, from 1600 h on day 2 until 0900 h on day 5.

#### *4.2.3.7 Dietary assessment*

Four-day food diaries, including 2 weekdays and 2 weekend days were collected prior to each experiment week. Instructions on how to complete the diary were provided at the screening visit.

#### *4.2.3.8 Data analysis*

The primary outcome measure of total energy intake over the three *ad libitum* eating days was analysed using a random effects model for continuous normal data. Subjects were the only random effect to make the analysis within subject; the fixed effects were the percentage of protein (categorical). In addition, period effects and the linear trend for percentage of protein were investigated. The primary comparisons of interest, 25%P vs. 15%P and 10%P vs. 15%P, were estimated with 95% confidence intervals.

Exploratory secondary endpoints were analysed using the same model. In addition, period-specific baseline measurements were used for body composition measurements to assess a carry-over effect. Appetite VAS scores and post-prandial blood concentrations were plotted over time. The area under the curve was calculated and analysed using the same random effects model.

Potential confounders were investigated by including them in the model as fixed effects and assessing whether the primary comparisons of interest remain qualitatively unchanged. All assumptions of the models were checked using Q-Q plot of residuals to check normality, residuals versus predicted values to check homogeneity of residual variance, residuals versus percentage protein to check for model fit.

## 4.3 Results

### 4.3.1 Subject Characteristics

Subjects were recruited from the general population. Fifteen subjects appeared eligible to participate in the study after completing the telephone-screening questionnaire and attended MRC HNR for a screening visit. Of these, two were ineligible because they exceeded the upper limit of BMI, and three were unable to commit adequate time for the study. The ten remaining subjects were recruited, and all subjects completed the three study visits.

Baseline characteristics of the study population are shown in Table 4.3.1. There were 5 male and 5 female subjects. The mean age was 43.4 y (SD 14.4, range 24-64) and mean BMI was 22.2 kg/m$^2$ (SD 2.2, range 17.5 – 24.4 kg/m$^2$). Mean waist circumference was 78 cm (SD 10.9, range 58 – 94). Mean EAT-26 was 3.7 (SD 2.8, range 0 – 9). All subjects performed some physical activity, but only six subjects engaged in vigorous exercise, with maximum reported exercise time of 39 min/day. Subjects reported spending 30.7 min/d (SD 27.9) engaged in moderate exercise and 36.2 min/d (SD 34.9) performing light activity.

There was no difference between male and female subjects in age ($p = 0.627$), BMI ($p = 0.051$), systolic ($p = 0.055$) or diastolic ($p = 0.212$) blood pressures, EAT-26 score ($p = 0.344$) or physical activity (vigorous $p = 0.46$, moderate $p = 0.74$, light $p = 0.64$, none $p = 0.13$). As would be expected, waist circumference was significantly lower in female (mean 69.8 cm, SD 6.9) than male (mean 86.2 cm, SD 7.2) subjects ($p = 0.006$). Given

the similarities between male and female subjects, the data were analysed as a single group.

**Table 4.3.1 Characteristics of the study population**

| n = 10 | Unit | Mean | SD | Range |
|---|---|---|---|---|
| **Age** | y | 43.4 | 14.4 | 24 - 64 |
| **Body Mass** | kg | 65.8 | 12.6 | 38.9 - 82.1 |
| **Height** | m | 1.71 | 0.10 | 1.49 - 1.84 |
| **BMI** | $kg/m^2$ | 22.2 | 2.2 | 17.5 - 24.4 |
| **Systolic BP** | mm Hg | 122 | 12 | 100 - 139 |
| **Diastolic BP** | mm Hg | 71 | 10 | 59 – 85 |
| **Waist Circumference** | cm | 78 | 10.9 | 58 – 94 |
| **EAT-26 score** | | 4 | 3 | 0 – 9 |
| **Physical Activity (from IPAQ)** | | | | |
| **- Vigorous** | min/day | 14.6 | 16.6 | 0 – 39 |
| **- Moderate** | | 30.7 | 27.9 | 0 – 77 |
| **- Light** | | 36.2 | 34.9 | 0 – 120 |
| **- None** | | 785 | 450 | 120- 1680 |

## 4.3.2 Baseline diets

There was no evidence of a difference within subjects in recorded food intake, using a four-day food diary, prior to each visit for energy intake ($p = 0.93$), protein ($p = 0.78$), fat ($p = 0.9$), carbohydrate ($p = 0.12$), NSP (non-starch polysaccharide fibre) ($p = 0.65$) or

alcohol (p = 0.06). There was no effect of visit order. Individual results are shown in Figure 4.3.1. Table 4.3.2 shows the mean energy intake prior to each study visit, and the percentage of energy consumed from protein, fat and carbohydrate.

**Figure 4.3.1 Individual macronutrient intake (as a % of EI) prior to each study visit**

C = carbohydrate intake, F = fat intake, P = protein intake

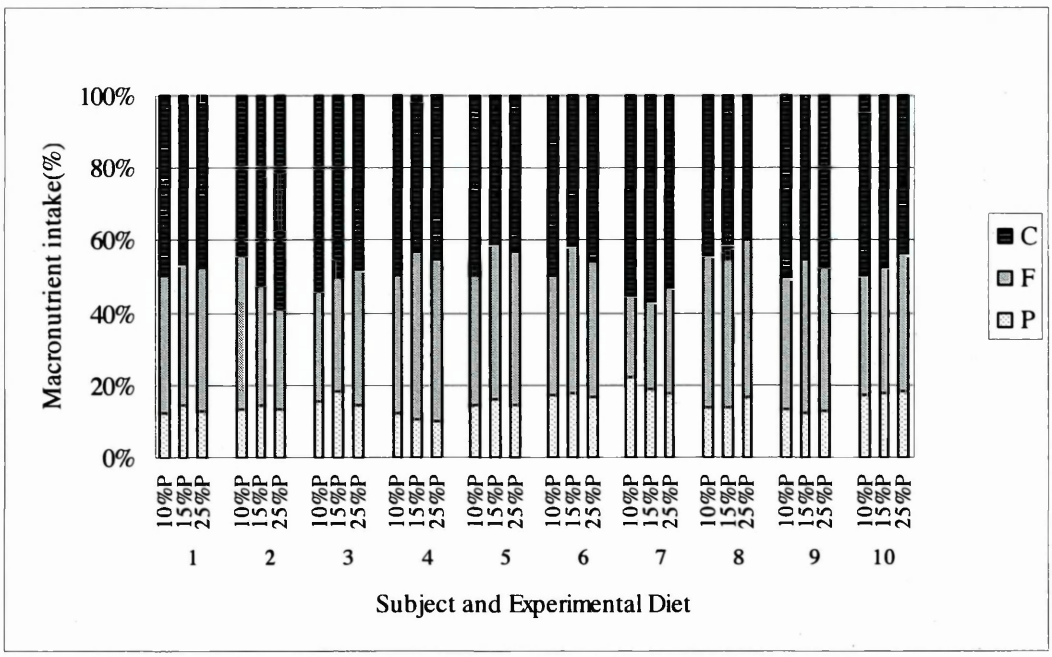



**Table 4.3.2 Recorded food intake from four-day food diaries compiled prior to the experimental visits (Mean, CI)**

| Food record prior to diet: | Energy Intake (MJ) | % of energy from protein | % of energy from fat | % of energy from carbohydrate |
|---|---|---|---|---|
| **10%P** | 8.4 (7.7, 9.7) | 15.2 (13.4, 17.0) | 35.4 (31.6, 39.1) | 50.0 (46, 53.6) |
| **15%P** | 8.6 (7.6, 9.6) | 15.4 (13.6, 17.2) | 37.7 (33.9, 41.5) | 47 (43.4, 50.8) |
| **25%P** | 8.6 (7.6, 9.6) | 14.4 (12.6, 16.2) | 37.5 (33.7, 41.3) | 47 (43.2, 50.6) |

### 4.3.3 Outcome measures

#### *4.3.3.1 Study diets*

Composition of the study diets was verified by measuring urine nitrogen, and palatability of the study diets was assessed to ensure the diets were of similar taste.

##### 4.3.3.1.1 Palatability

There was little difference between subjects' rating of palatability of the study diets and no difference between diets in the overall mean palatability scores for each question on the palatability questionnaire (Table 4.3.3).

The 25%P breakfast was rated more filling than the 10%P ($p = 0.008$) or 15%P ($p = 0.027$) breakfasts but subjects found the 15%P breakfast more enjoyable than the 25%P ($p = 0.03$). The 25%P lunch main meal was rated tastier than the 10%P ($p = 0.005$) and more satisfying compared to the 10%P ($p = 0.003$) and 15%P ($p = 0.02$) diets. There was no difference in palatability ratings for the lunch dessert. The dinner main meal scored higher for tastiness with 15%P than 10%P ($p = 0.011$) and was more pleasant ($p = 0.012$). The 25%P ($p = 0.008$) and 15%P ($p = 0.044$) meals were more enjoyable than the 10%P meal. There were no differences in ratings for the dinner dessert.

**Table 4.3.3 Mean (SEM) VAS scores for palatability of the study meals**

| Meals | Sweet | Tasty | Savoury | Pleasant | Filling | Satisfying | How much more? | Enjoyable |
|---|---|---|---|---|---|---|---|---|
| All meals | | | | | | | | |
| 10% | 52 ± 6 | 65 ± 6 | 39 ± 6 | 67 ± 6 | 72 ± 4 | 70 ± 6 | 11 ± 2 | 65 ± 6 |
| 15% | 45 ± 6 | 68 ± 6 | 40 ± 6 | 70 ± 6 | 75 ± 4 | 68 ± 6 | 12 ± 2 | 69 ± 6 |
| 25% | 47 ± 6 | 67 ± 6 | 38 ± 6 | 68 ± 6 | 78 ± 4 | 73 ± 6 | 10 ± 2 | 67 ± 6 |
| Breakfast | | | | | | | | |
| 10% | 54 ± 8 | 72 ± 6 | 24 ± 7 | 73 ± 6 | 73 ± 5 * | 74 ± 5 | 12 ± 4 | 71 ± 6 |
| 15% | 56 ± 8 | 69 ± 6 | 32 ± 7 | 74 ± 6 | 75 ± 5 § | 77 ± 5 | 14 ± 4 | 77 ± 6 § |
| 25% | 60 ± 8 | 65 ± 6 | 24 ± 7 | 64 ± 6 | 87 ± 5 | 81 ± 5 | 10 ± 4 | 66 ± 6 |
| Lunch main | | | | | | | | |
| 10% | 23 ± 8 | 45 ± 10 * | 65 ± 9 | 50 ± 9 * | 69 ± 6 * | 53 ± 9 * | 7 ± 3 | 44 ± 10 * |
| 15% | 16± 8 | 52 ± 10 | 63 ± 9 | 52 ± 9 § | 78 ± 6 | 58 ± 9 § | 7 ± 3 | 53 ± 10 |
| 25% | 22 ± 8 | 67 ± 10 | 66 ± 9 | 70 ± 9 | 82 ± 6 | 74 ± 9 | 8 ± 3 | 60 ± 10 |

| Meals | Sweet | Tasty | Savoury | Pleasant | Filling | Satisfying | How much more? | Enjoyable |
|---|---|---|---|---|---|---|---|---|
| Lunch dessert | | | | | | | | |
| 10% | 81 ± 6 | 79 ± 6 | 28 ± 8 | 78 ± 5 | 73 ± 7 | 81 ± 8 | 13 ± 5 | 77 ± 7 |
| 15% | 63 ± 6 | 79 ± 6 | 20 ± 8 | 80 ± 5 | 71 ± 7 | 68 ± 8 | 16 ± 5 | 78 ± 7 |
| 25% | 69 ± 6 | 69 ± 6 | 16 ± 8 | 73 ± 5 | 72 ± 7 | 70 ± 8 | 14 ± 5 | 72 ± 7 |
| Dinner main | | | | | | | | |
| 10% | 22 ± 8 | 55 ± 8 | 59 ± 10 | 60 ± 8 # | 70 ± 7 | 67 ± 7 | 9 ± 3 | 56 ± 8 # * |
| 15% | 19 ± 8 | 76 ± 8 | 70 ± 10 | 76 ± 8 | 78 ± 7 | 74 ± 7 | 10 ± 3 | 70 ± 8 |
| 25% | 16 ± 8 | 69 ± 8 | 69 ± 10 | 71 ± 8 | 70 ± 7 | 74 ± 7 | 10 ± 3 | 75 ± 8 |
| Dinner dessert | | | | | | | | |
| 10% | 74 ± 6 | 74 ± 7 | 23 ± 7 | 75 ± 7 | 76 ± 5 | 74 ± 8 | 12 ± 4 | 75 ± 8 |
| 15% | 70 ± 6 | 65 ± 7 | 18 ± 7 | 69 ± 7 | 74 ± 5 | 65 ± 8 | 10 ± 4 | 70 ± 8 |
| 25% | 65 ± 6 | 66 ± 7 | 18 ± 7 | 64 ± 7 | 78 ± 5 | 67 ± 8 | 8 ± 4 | 60 ± 8 |

* 25%P diet different from 10%P, § 25%P diet different from 15%P, # 15%P diet different from 10%P

4.3.3.1.2 Urinary nitrogen

Nitrogen excretion during the 25%P diet (0.77% ± 0.07) was significantly higher than during the 15%P diet (0.49% ± 0.07) (P<0.0001) and the 10%P diet (0.40%±0.07) (P <0.0001) (Figure 4.3.2). The difference between the 10%P and 15%P diets was not significant (P = 0.18). Individual results are shown in Figure 4.3.3. There was no effect of visit order on nitrogen excretion rates. Unfortunately three of the samples were misplaced during storage at the CRF and were not able to be included in this analysis.

**Figure 4.3.2 Mean (± SEM) urine nitrogen collected on day 4 of each study visit**

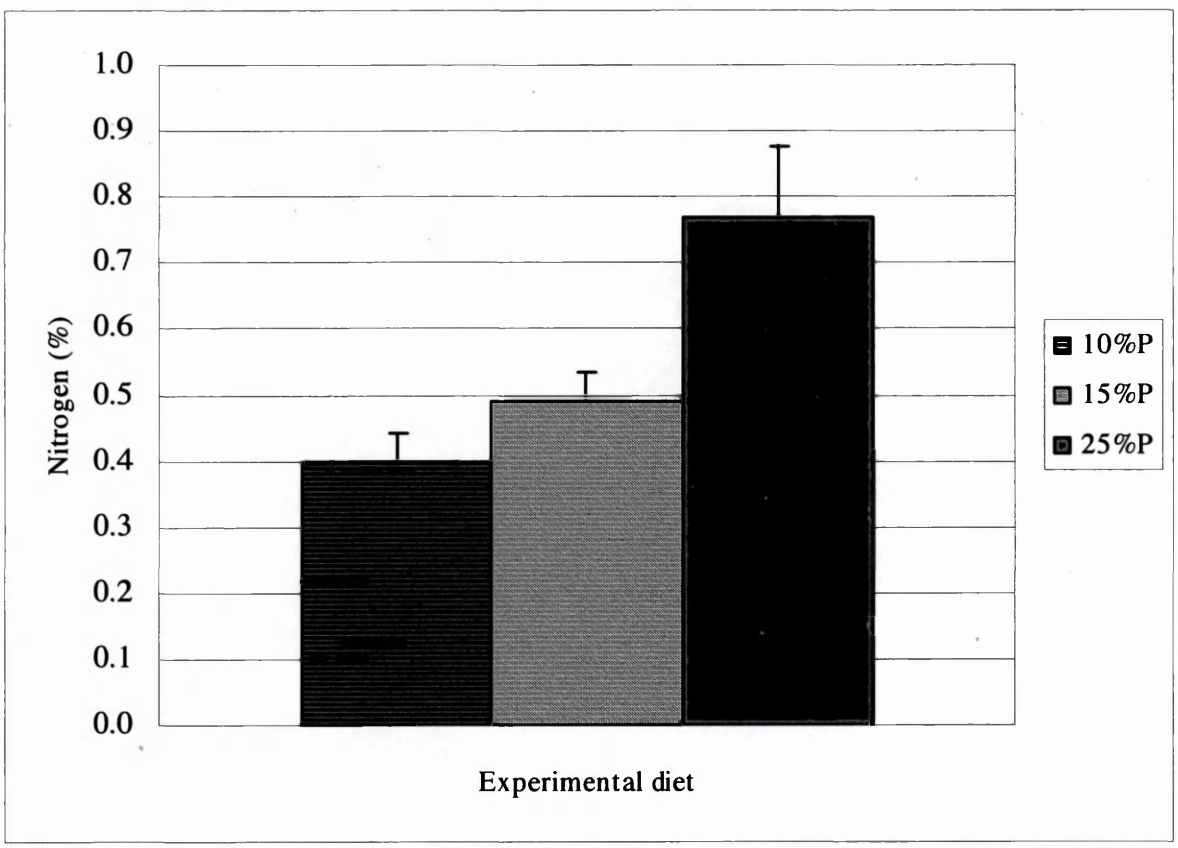
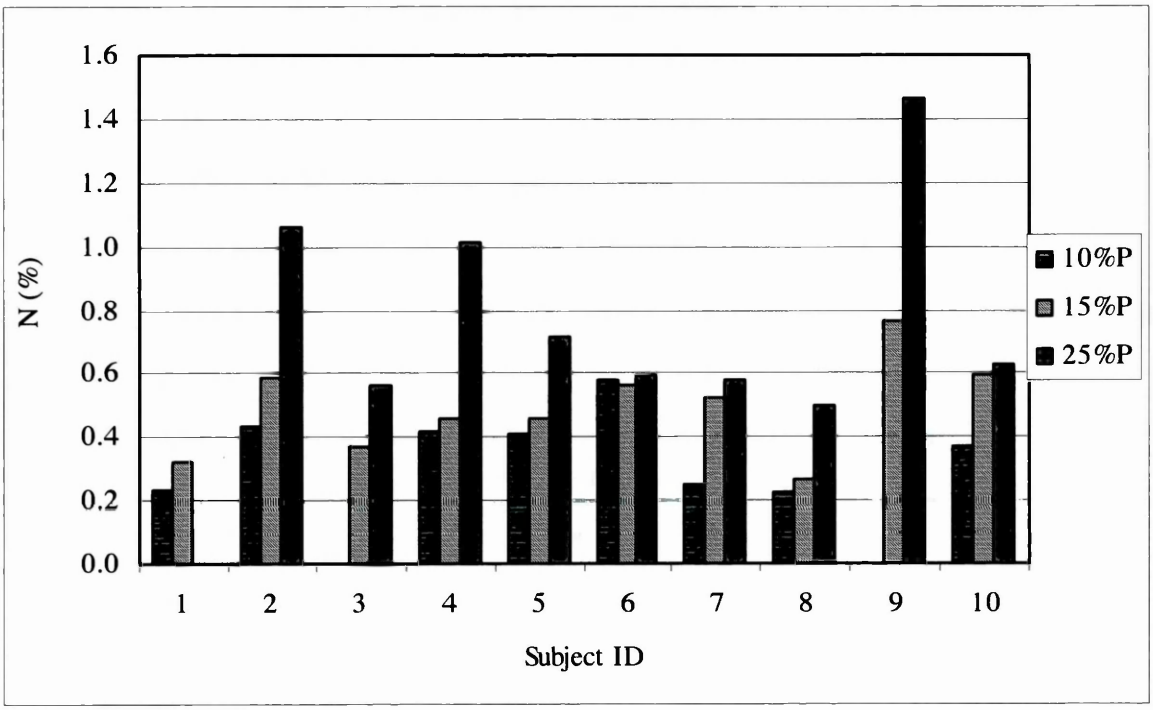


**Figure 4.3.3 Urinary nitrogen excretion on day 4 of each study visit for individual subjects.**

10%P n = 8, 15%P n = 10, 25%P n = 9,

no data was available for subject 1, 25%P; subject 3, 10%P; and subject 9, 10%P




*4.3.3.2 Energy intake*

Energy intake (EI) was analysed by comparing the three study diets, and separated into energy intake per day, per meal, and eating occasion (meals versus snacks). All statistical analyses were performed twice, including and excluding subject 8, whose energy intake exceeded requirements by 52 – 84% on each treatment. Results reported include all subjects unless removing subject 8 changed the outcome of the analysis, where both results are included.

4.3.3.2.1 Total EI per diet

Individual total EI per treatment is shown in Figure 4.3.4. With all subjects included in the analysis the total energy intake was not significantly different between each study diet ($p = 0.16$). The mean total EI during the three *ad libitum* days on the 10%P diet was 28.5

MJ/3 days (SD 13.3), during the 15%P diet was 28.2 MJ/3 days (SD 11.1) and during the 25%P diet was 27.0 MJ/3 days (SD 12.0) (Figure 4.3.4) When subject 8 was excluded from the analysis the mean total EI during the 25%P diet (24.2 MJ/3 d, SD 8.7, 8.1MJ/d, SE 1.0) was significantly lower than the 15%P visit (26.1 MJ/3 d, SD 9.2, 8.7 MJ/d, SE 1.0), ($p = 0.001$), with a mean of 25.3 MJ/3 days, SD 9.3, 8.4 MJ/d, SE 1.0) during the 10%P diet (Figure 4.3.5). The difference was not affected by order of subjects' visits. There was no difference in energy intake between the diets when analysed per meal or per day.

**Figure 4.3.4 Total energy intake (EI) over three experimental days for all subjects for each experimental diet**

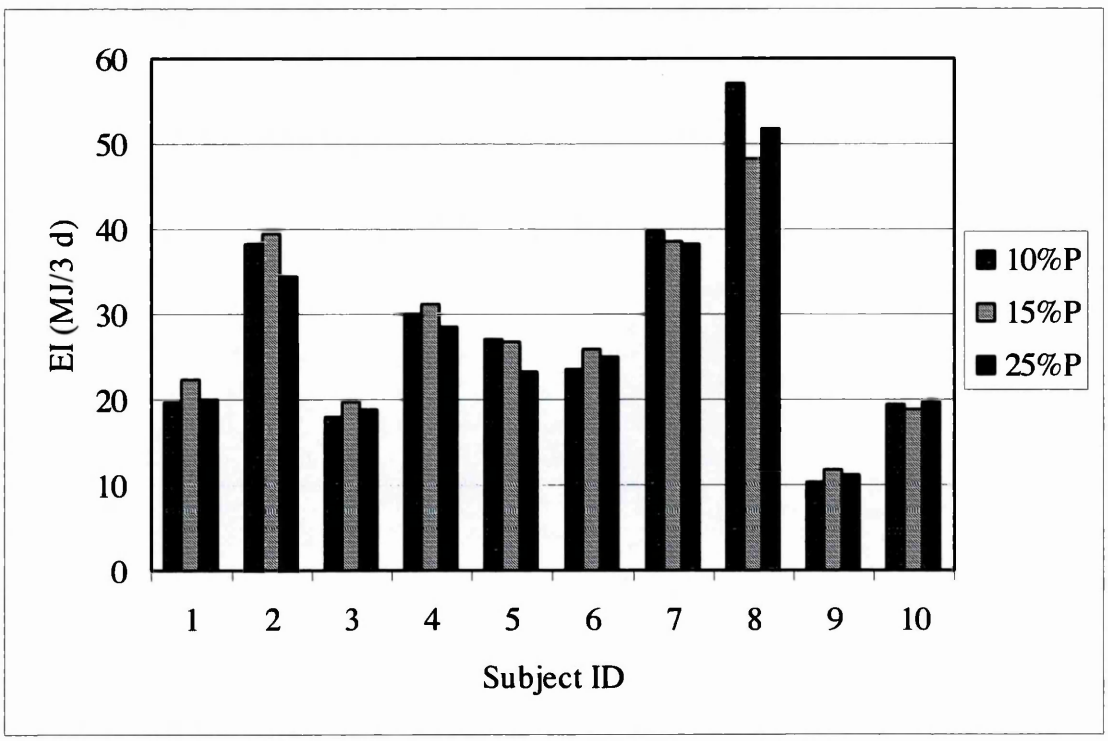
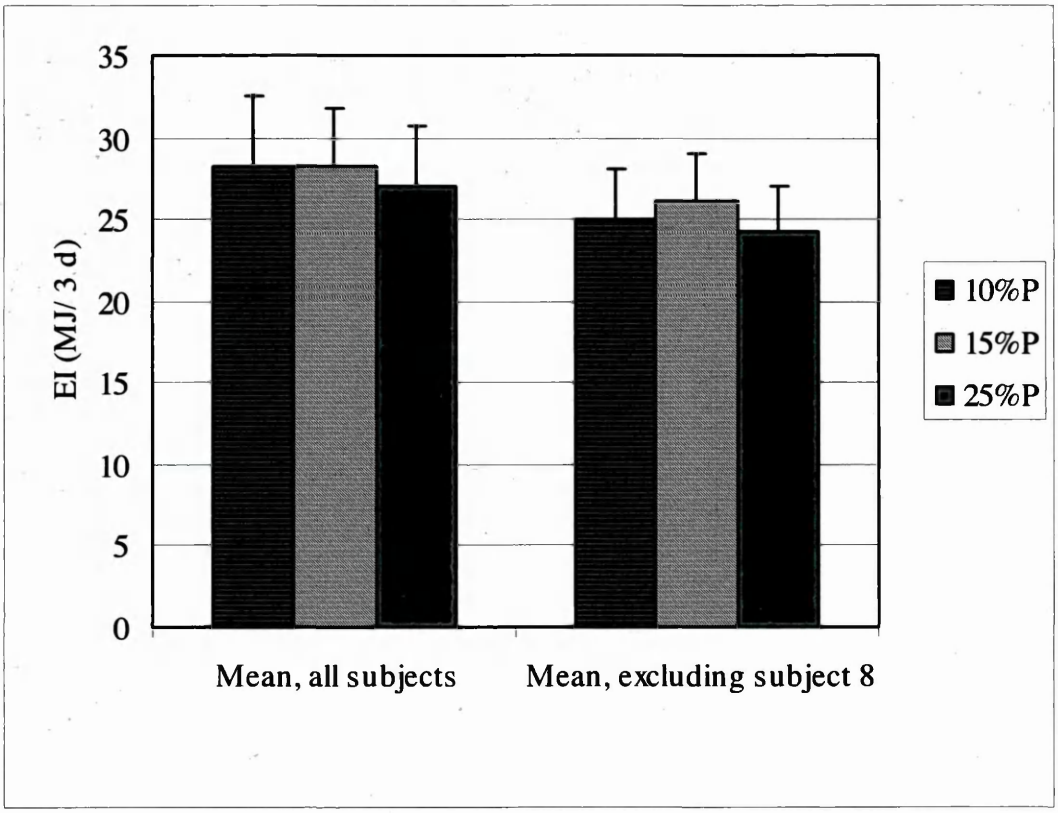
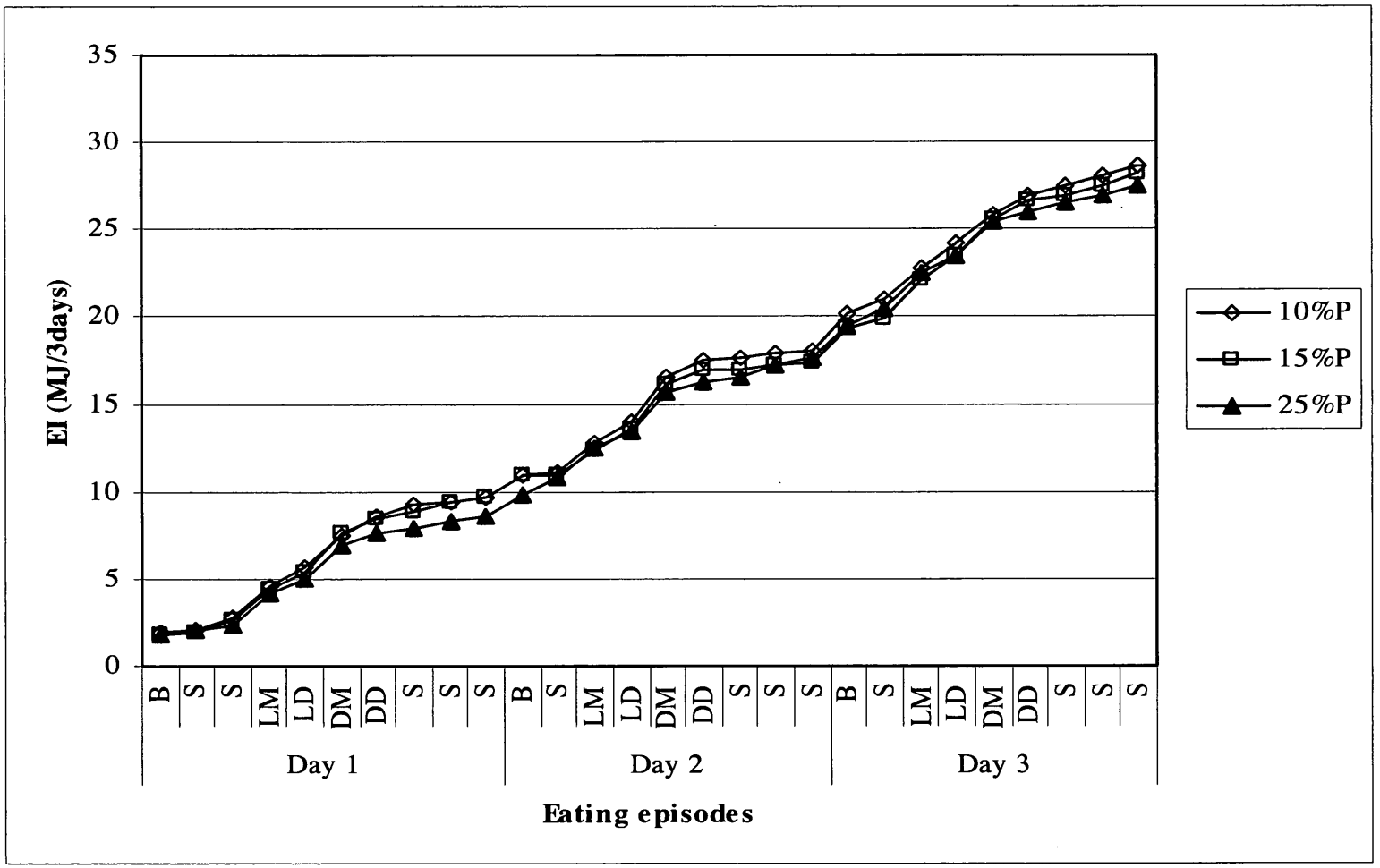


**Figure 4.3.5 Mean energy intake for all subjects and the cohort excluding subject 8 for each experimental diet**



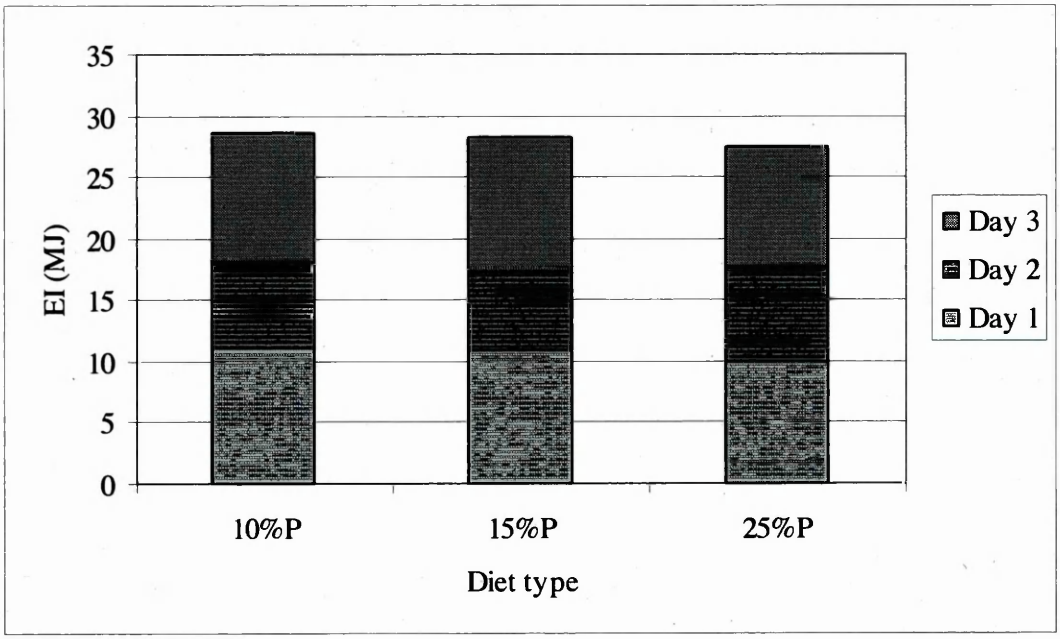

Cumulative energy intake over the three days was plotted for each eating episode and the mean results are presented in Figure 4.3.6 and Figure 4.3.7. Figure 4.3.6 shows that food intake pattern was very similar for each day, with slightly lower intake during the 25%P diet throughout the entire three-day experiment, particularly towards the end of each day (dinner and the evening snacks). This effect was evident from day 1 of the study. Total cumulative energy intake is presented in Figure 4.3.7 for each study diet and demonstrates the similarity in the pattern of consumption for each day of the study visits.

**Figure 4.3.6 Mean cumulative energy intake for all subjects for each eating episode over the three experimental days**




B = breakfast, S = snack, LM = lunch main, LD = lunch desert, DM = dinner main, DD = dinner desert

**Figure 4.3.7 Mean cumulative energy intake for all subjects for the three experimental days of each study visit.**




4.3.3.2.2 EI per diet per day

Data were separated into different time periods throughout the experimental week to look in greater detail at the pattern of EI. The EI per day of each study visit is shown in Figure 4.3.8 for individual subjects and the mean is shown in Figure 4.3.9. There was a significant effect of diet and day on energy intake ($p = 0.0015$). On the 10%P diet, EI was lower on day 2 than day 1 or 3 ($p = 0.015$). On the 15%P diet, EI was lower on day 2 than day 1 ($p < 0.0001$) with the greatest intake on day 3 ($p = 0.028$). During the 25%P diet, EI did not differ between day 1 and 2 but was marginally higher on day 3 than day 1 ($p = 0.05$).

**Figure 4.3.8 EI on each study day for all subjects for each experimental diet**

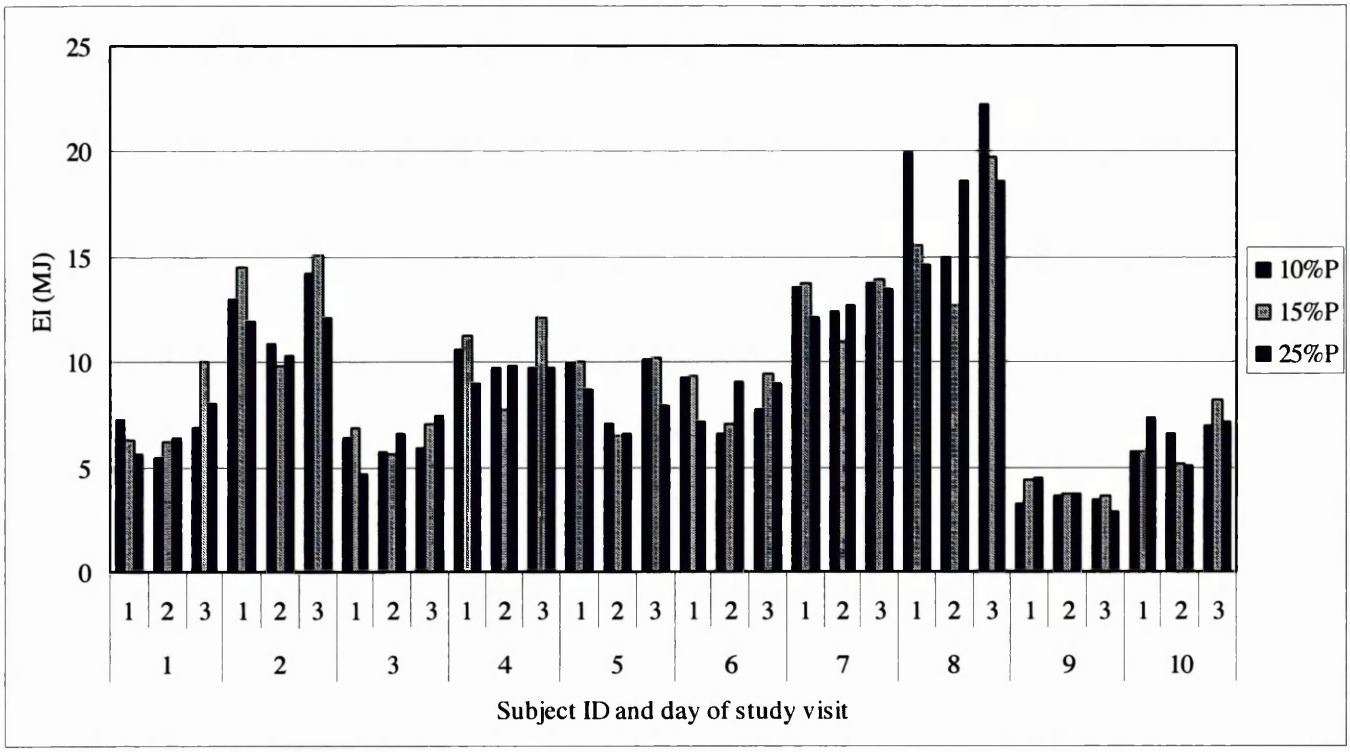
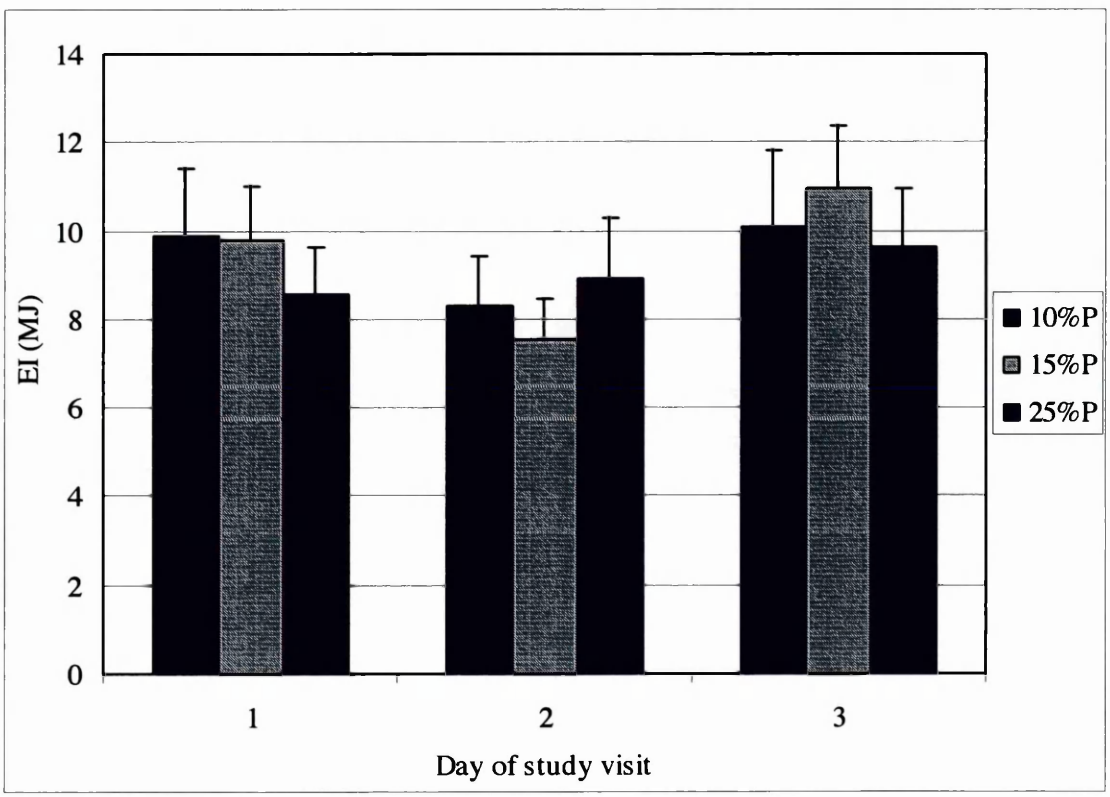



**Figure 4.3.9 Total Mean (SEM) EI per day for the three *ad libitum* days of each study diet**

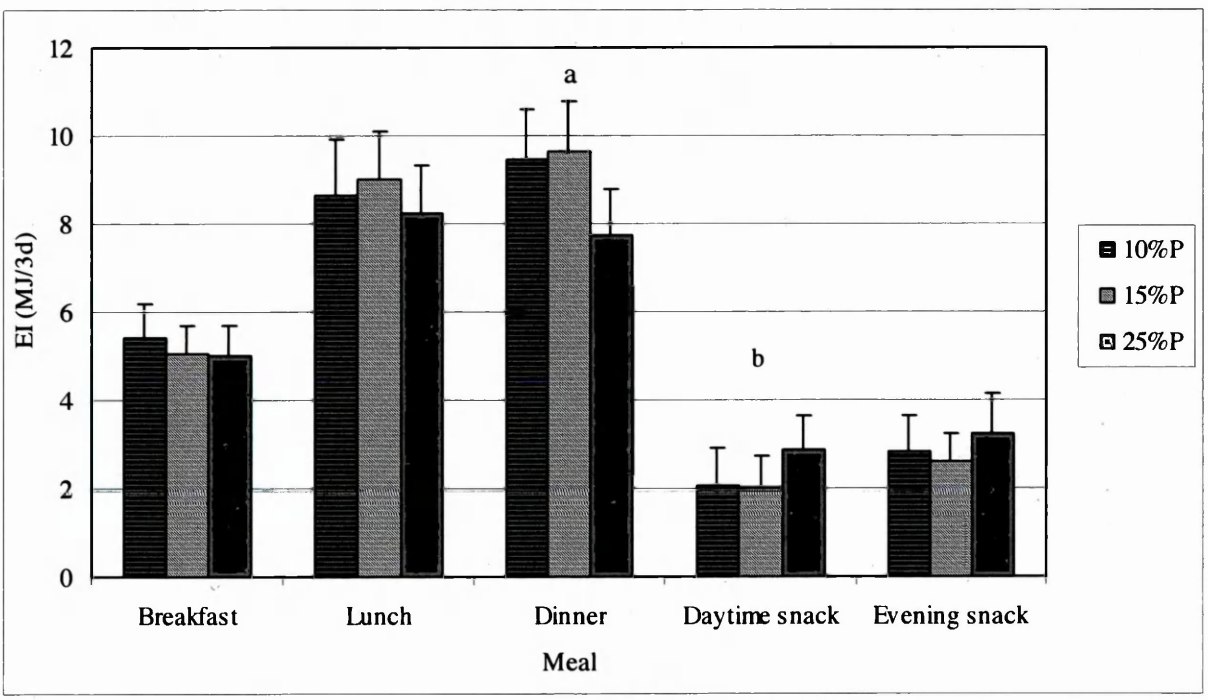


4.3.3.2.3 EI per meal per diet

EI between diets was no different for breakfast (p = 0.29), lunch (p = 0.20), or evening snacks (p = 0.40). EI at dinner was significantly reduced during the 25%P diet compared to the 10%P and 15%P diets (p = 0.0002). Day snacks (DS) were significantly increased during the 25%P diet compared to the 10%P and 15%P diets (p = 0.01). When subject 8 was removed from the analysis, there was an additional difference in EI at lunch which was significantly lower during the 10%P (p = 0.04) and 25%P diet (p = 0.015) compared to the 15%P diet. Individual EI for each meal is shown in Figure 4.3.11. The mean total EI for each meal is shown in Figure 4.3.10. Whilst EI intake differed between meals (p < 0.00001) there was no diet and meal interaction for EI (p = 0.220).

**Figure 4.3.10 Mean (SEM) total EI per meal per diet over the 3 study days**

a, 25%P < 15%P and 10%P
b, 25%P > 15%P and 10%P



EI was also analysed by separating food consumption into meals (including all food consumed at breakfast, lunch and dinner) or snacks (daytime and evening snacks). During the 25%P diet total energy intake from meals was reduced (20.9 MJ/3 d) ($p = 0.0001$) compared with the 15%P (23.7 MJ/3 d) and 10%P (23.5 MJ/3 d) diets. Conversely EI from snacks was significantly increased during the 25%P diet (6.1 MJ/3d) compared with the 15%P (4.6 MJ/3d, $p = 0.001$) and 10%P (4.8 MJ/3d, $p = 0.004$) diets. EI per meal type is shown in Figure 4.3.12 for individual subjects and the mean is shown in Figure 4.3.13. As expected, EI from meals was significantly higher than from snacks ($p = 0.00001$).

**Figure 4.3.11 Total EI per meal per diet for individual subjects**

B = Breakfast, L = Lunch, D = Dinner, DS = Day Snack, ES = Evening Snack

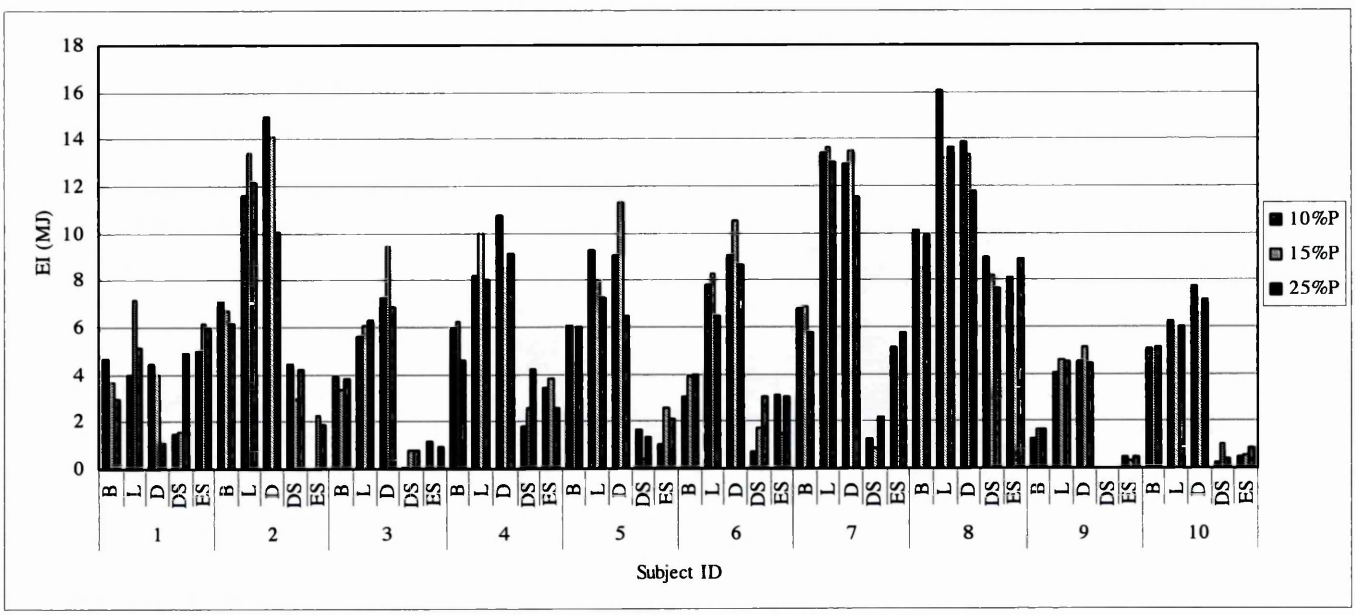


**Figure 4.3.12 Total EI per meal type per subject, M = meal, S = snack**

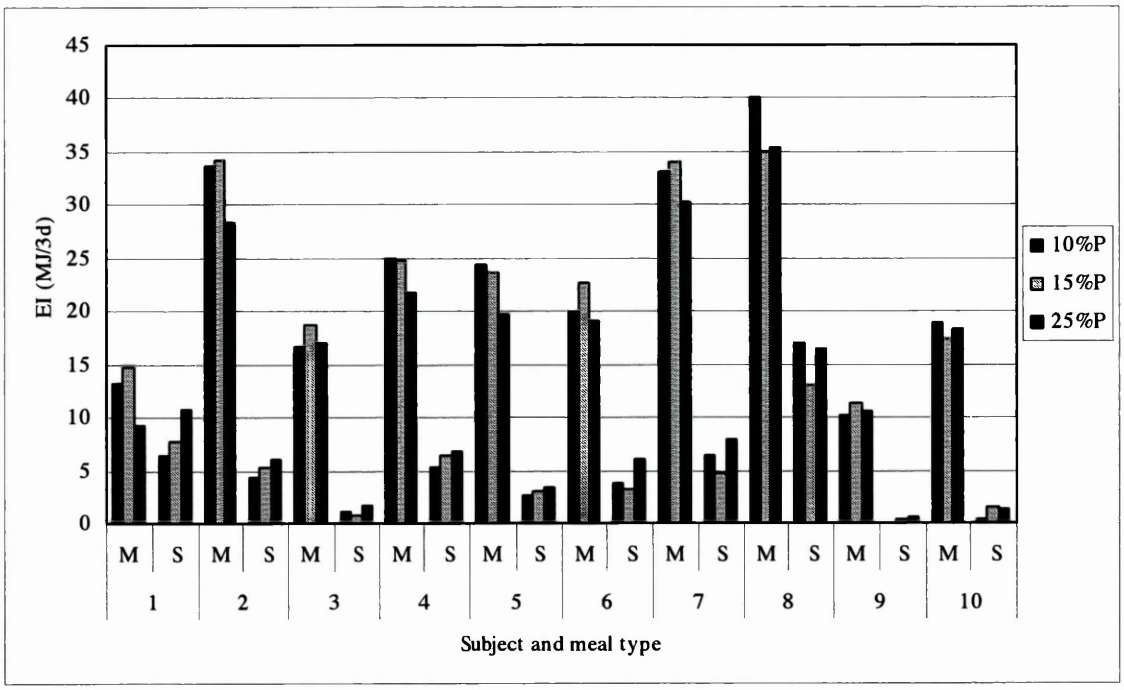
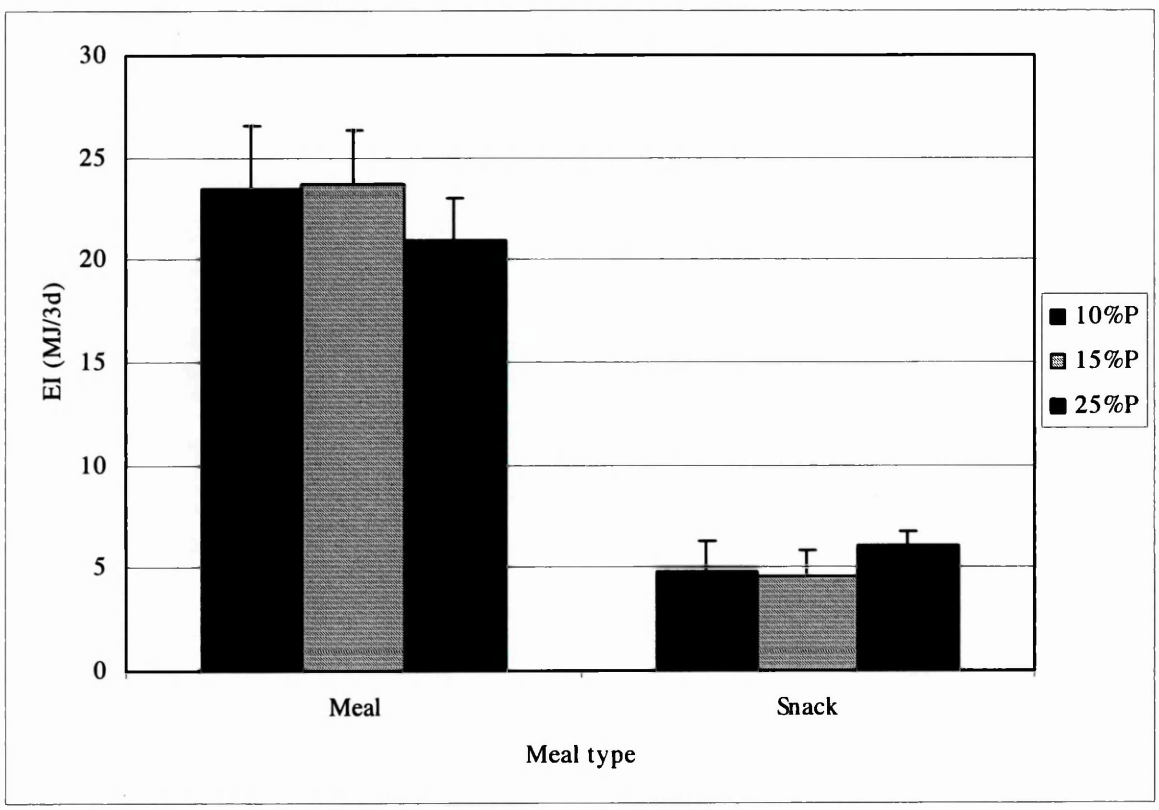



**Figure 4.3.13 Mean (SEM) total EI per meal type**

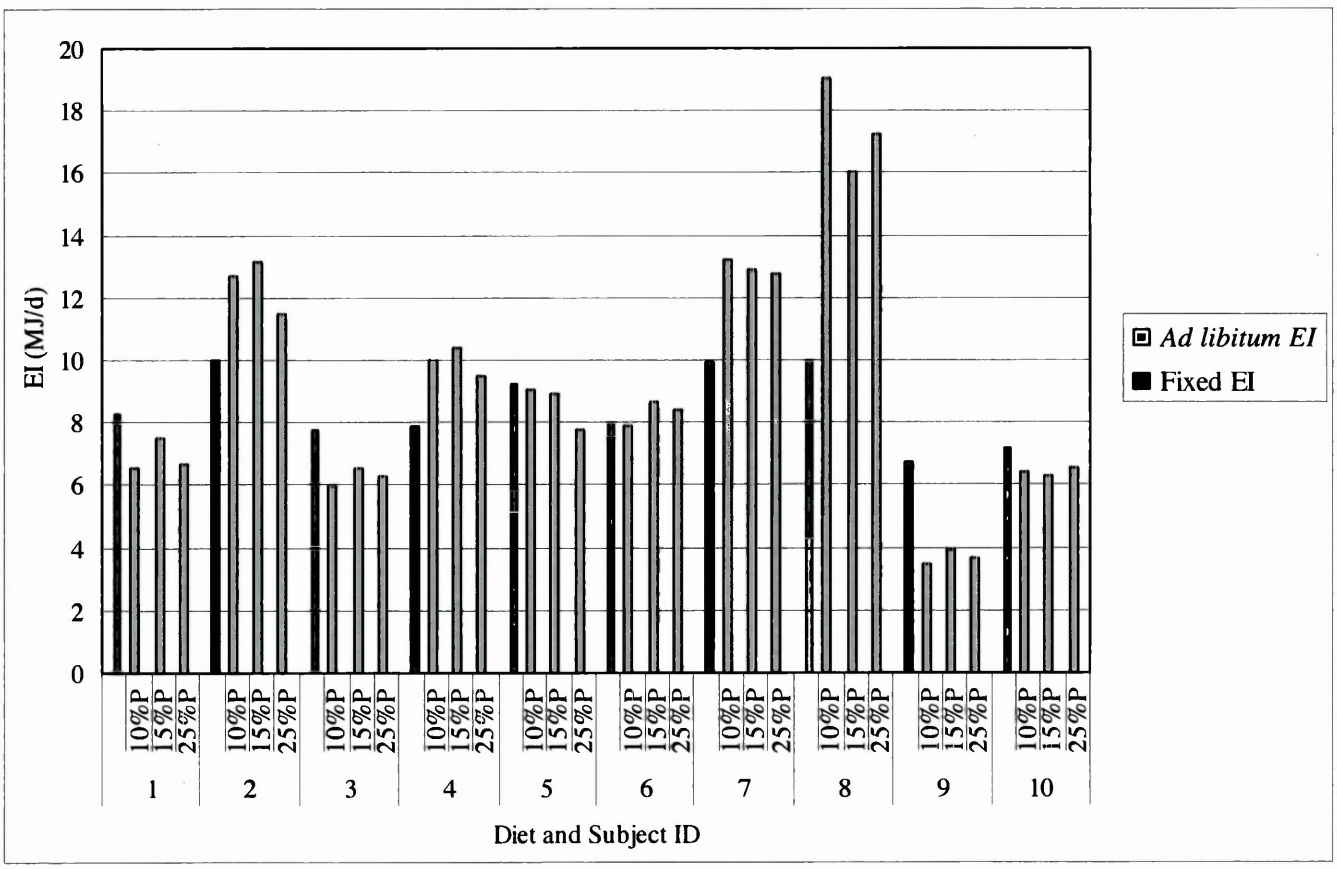


4.3.3.2.4 *Ad libitum* EI versus calculated energy requirements

Energy intake during the *ad libitum* eating days was compared to the energy of food provided during the fixed-eating day. As detailed in Chapter 5 the calculation was based on measured BMR, with an allowance for the two periods of exercise that were performed whilst in the calorimeter. The total exercise was similar to the one-hour walk that subjects performed each *ad libitum* day, so the calculated energy intake should approximate energy needs on all study days. Subjects were consistent in whether their EI during the *ad libitum* days exceeded energy requirements or was below requirements (Figure 4.3.14).

**Figure 4.3.14 EI during the *ad libitum* EI study days (day 1-3) and the calculated energy requirements (fixed EI day 4).**

Subjects 1, 3, 4, 9, 10 were female, subjects 2, 5, 6, 7, 8 were male

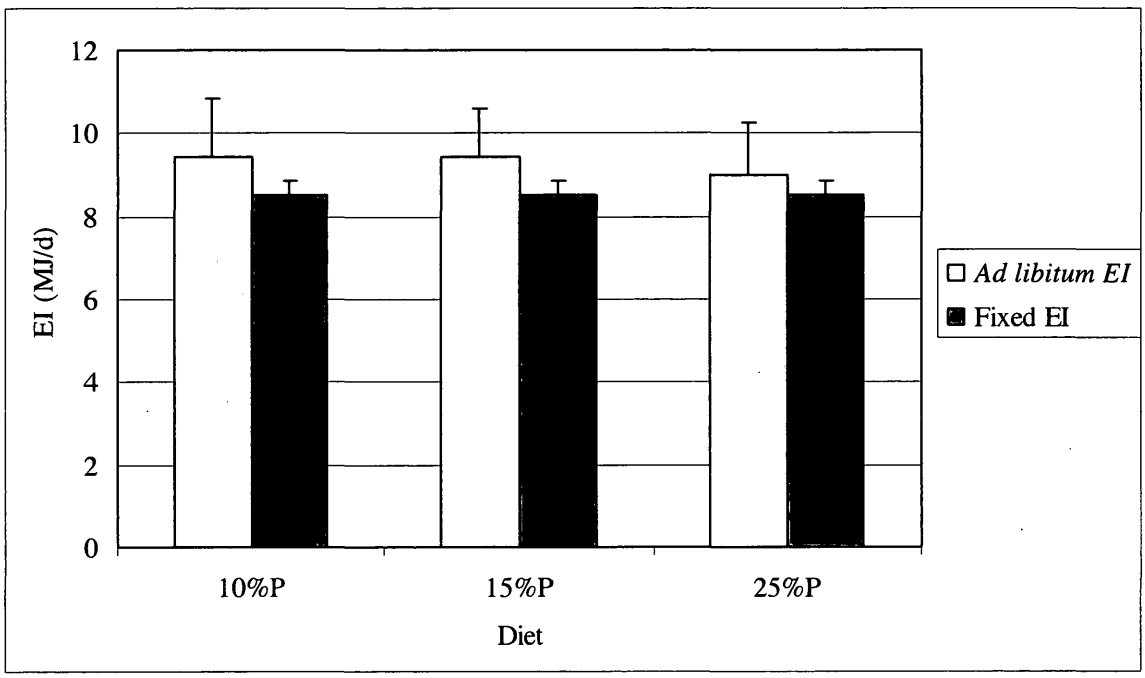


It is evident in this figure that subject 8 exceeded energy requirements by 1.5 – 2 times. When subject 8 was excluded from the analysis the mean difference in energy intake between the *ad libitum* eating days and the calculated energy requirements was small (Table 4.3.4). Excluding subject 8, EI was less than estimated energy needs during the 25%P diet, and EI was significantly lower during the 25%P diet than the 15%P diet ($p = 0.003$). Of the subjects who overate all except one was male, while all female subjects under-ate except one. Mean EI and the mean calculated energy requirement is shown in Figure 4.3.15.

**Table 4.3.4 Mean difference in EI between the *ad libitum* eating days and estimated energy requirements (excluding subject 8)**

| **Diet** | **Difference in energy intake (MJ)** | **SEM** |
|---|---|---|
| 10%P | 0.03 | 0.7 |
| 15%P | 0.4 | 0.7 |
| 25%P | -0.2 | 0.6 |

**Figure 4.3.15 Mean (SD) EI during the *ad libitum* study days (EI 123) and the calculated EI requirements (EI 4).**




*4.3.3.3 Hunger and satiety*

Fullness scores were significantly higher during the 25% P diet (p = 0.006) than the 15%P diet. Figure 4.3.17 demonstrates that scores were higher during the 25%P diet at 1100 h compared to the 10% P diet (p = 0.018), at 2000 h compared to the 10% P diet (p = 0.005), at 2100 h compared to the 10%P and 15%P diets (p = 0.019), and at 2200 h 25%P compared to the 15%P diet (p = 0.01). The AUC fullness scores were significantly higher during the 25%P diet than the 15%P (p = 0.001) and 10%P diets (p = 0.018) (Table 4.3.5). There was no difference in the maximum score (p = 0.54), time to maximum score (p = 0.77), or the minimum score (p = 0.36), but the minimum score was earlier in the day during the 25%P diet (p = 0.017) with most subjects reporting their lowest scores before breakfast.

There was no difference in self-reported hunger scores at any timepoint during the day (Figure 4.3.16) or for the AUC (Table 4.3.5).

The 15%P diet produced the highest scores for the question 'How much food do you think you could eat?' at 1100 h (compared to the 10%P and 25%P diets, $p = 0.033$), 1800 h (compared to the 10%P and 25%P diets, $p = 0.005$), and 2100 h (compared to the 25%P diets, $p = 0.04$) (Figure 4.3.18). There was no difference in the AUC (Table 4.3.5) or any other parameters.

In response to the question 'How strong is your desire to eat?' there was no difference in scores between the diets ($p = 0.89$) or at any timepoint during the day (Figure 4.3.19). The AUC did not differ between the diets (Table 4.3.5).

Self-reported scores for 'How content are you?' did not differ at any timepoint (Figure 4.3.20) or for the AUC (Table 4.3.5). Scores were higher for visit 2 ($p = 0.018$) and 3 ($p = 0.022$) than for visit 1.

In response to the question 'How irritable are you?' mean VAS scores were lower with the 15%P diet than 10%P ($p = 0.05$) or 25%P diets ($p = 0.006$). Figure 4.3.21 demonstrates that there was no difference in scores at specific timepoints during the day, and the AUC did not differ between diets (Table 4.3.5). Scores were lower during visits 2 and 3 ($p < 0.0001$).

Mean VAS scores were lower during the 15%P and 25%P diets than the 10 %P diets ($p < 0.0001$) for the question 'How depressed are you?'. Scores were lower for visit 2 than

visit 1 ($p < 0.0001$) but did not differ at any timepoint (Figure 4.3.22) or for the AUC (Table 4.3.5).

In response to the question 'How alert are you?' mean scores were higher during the 25%P diet than 10%P or 15%P diets ($p < 0.0001$) with higher scores at 1100 h ($p = 0.05$) and at 2200 h ($p = 0.04$) (Figure 4.3.23). Scores were lower during visit 3 than visit 1 ($p = 0.005$). There was no difference in the AUC (Table 4.3.5) or for any other parameter analysed.

**Figure 4.3.16 Mean ± SEM VAS score 'How hungry are you?'**

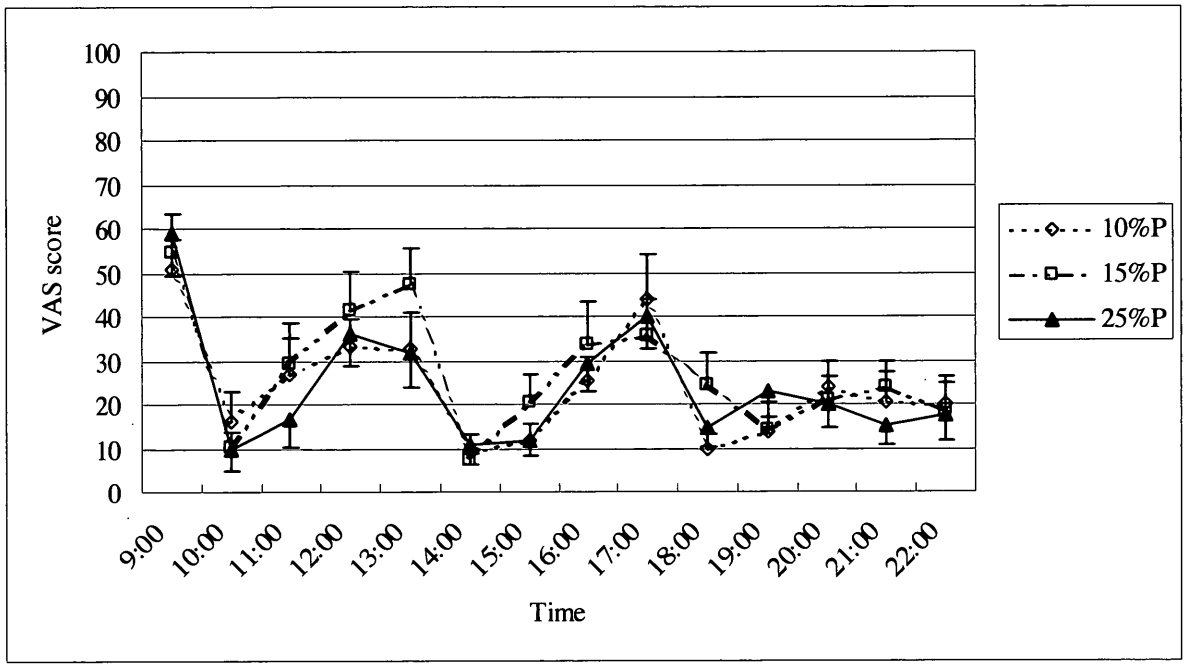
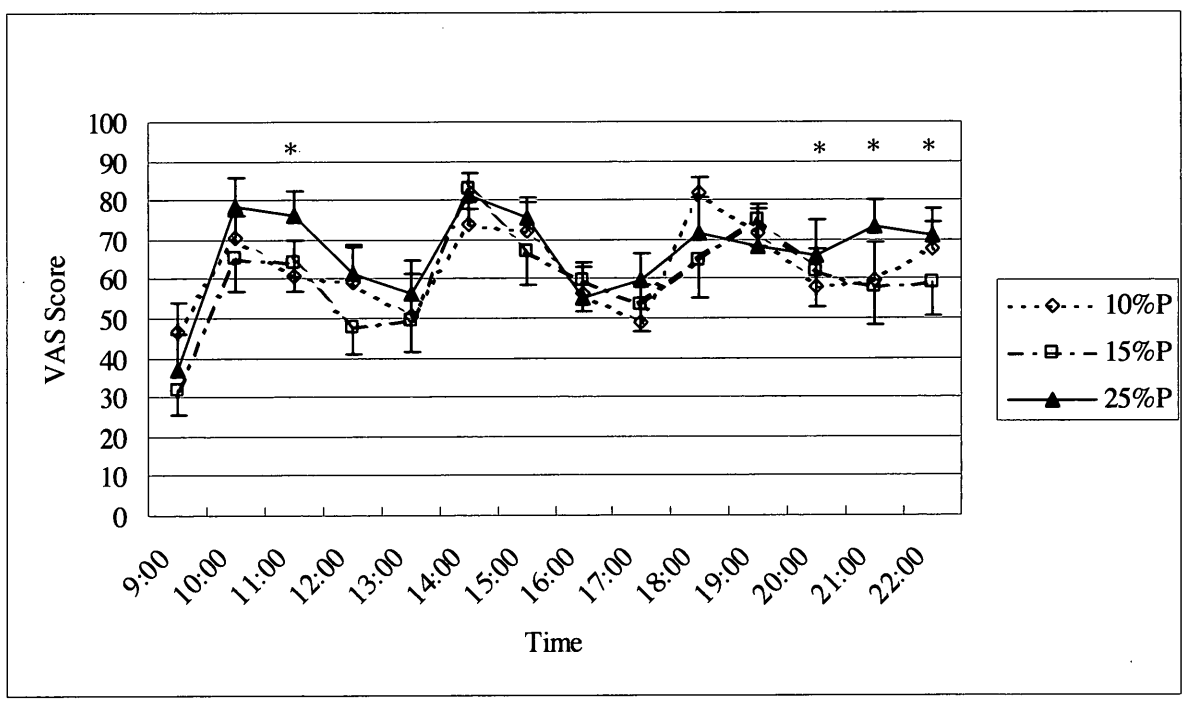



**Figure 4.3.17 Mean ± SEM VAS score 'How full do you feel?'**




* indicates significant difference between scores

**Figure 4.3.18 Mean ± SEM VAS score 'How much food do you think you could eat?'**

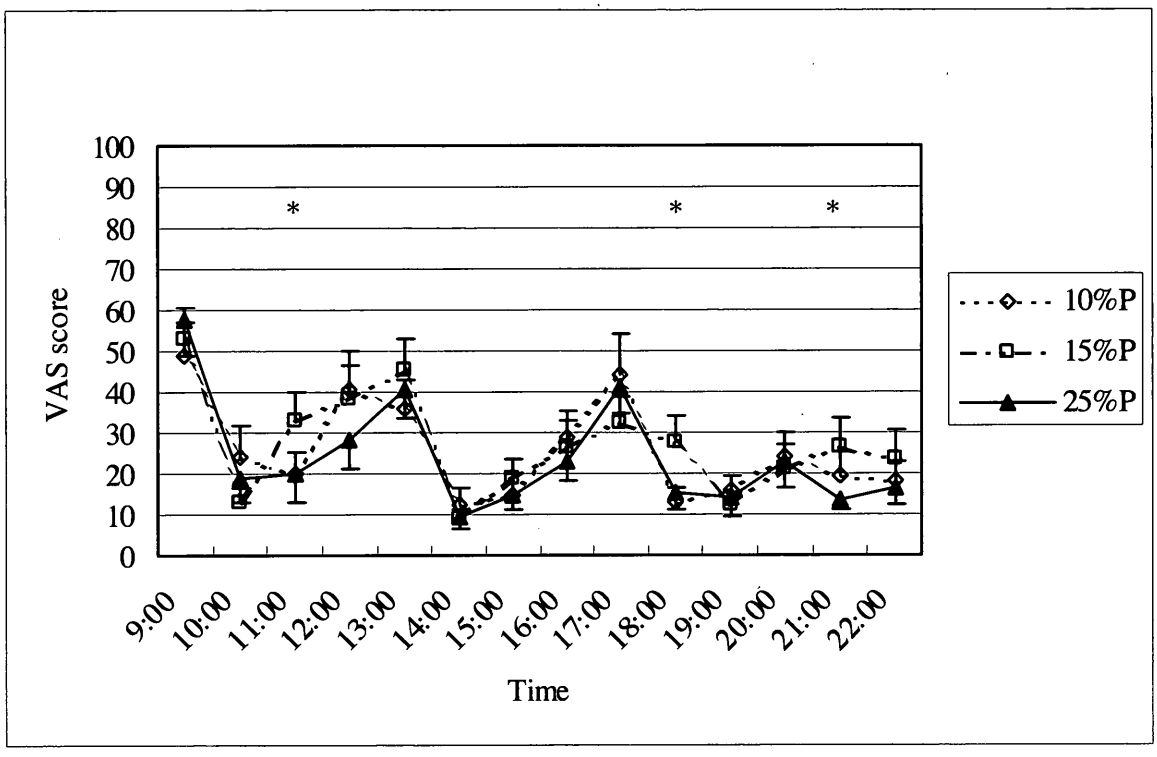



**Figure 4.3.19 Mean ± SEM VAS score 'How strong is your desire to eat?'**

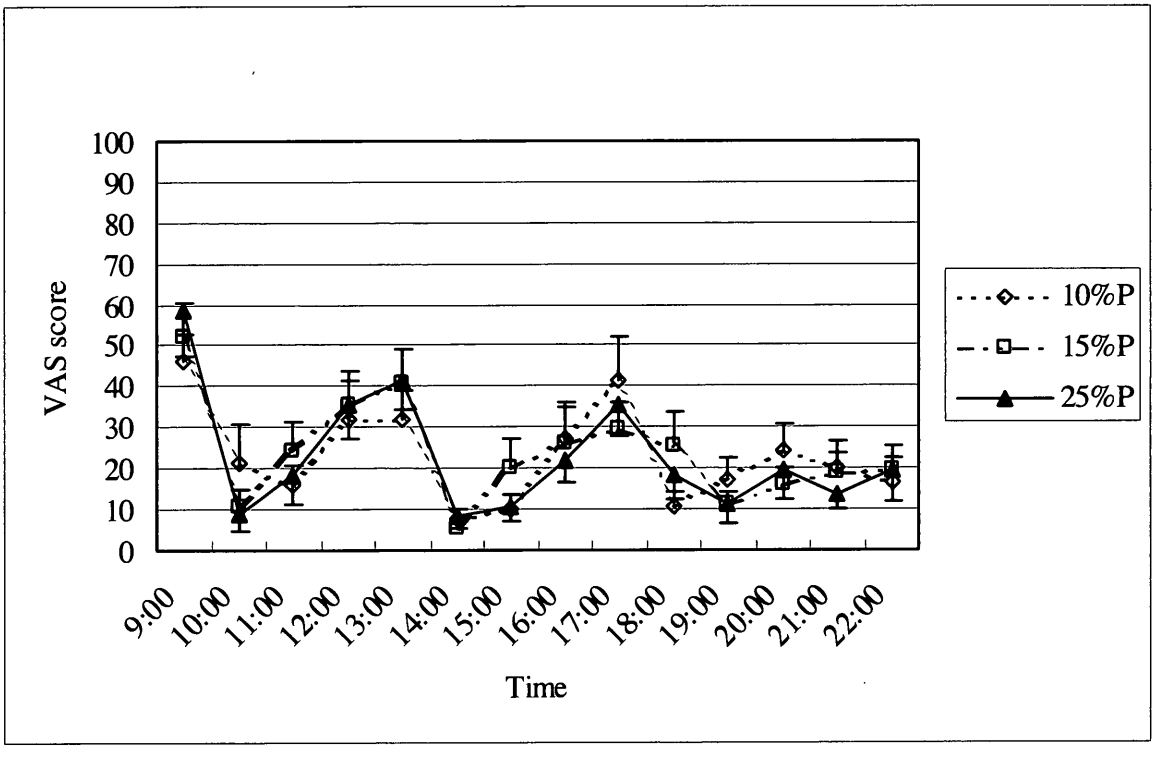


**Figure 4.3.20 Mean ± SEM VAS score 'How content are you?'**

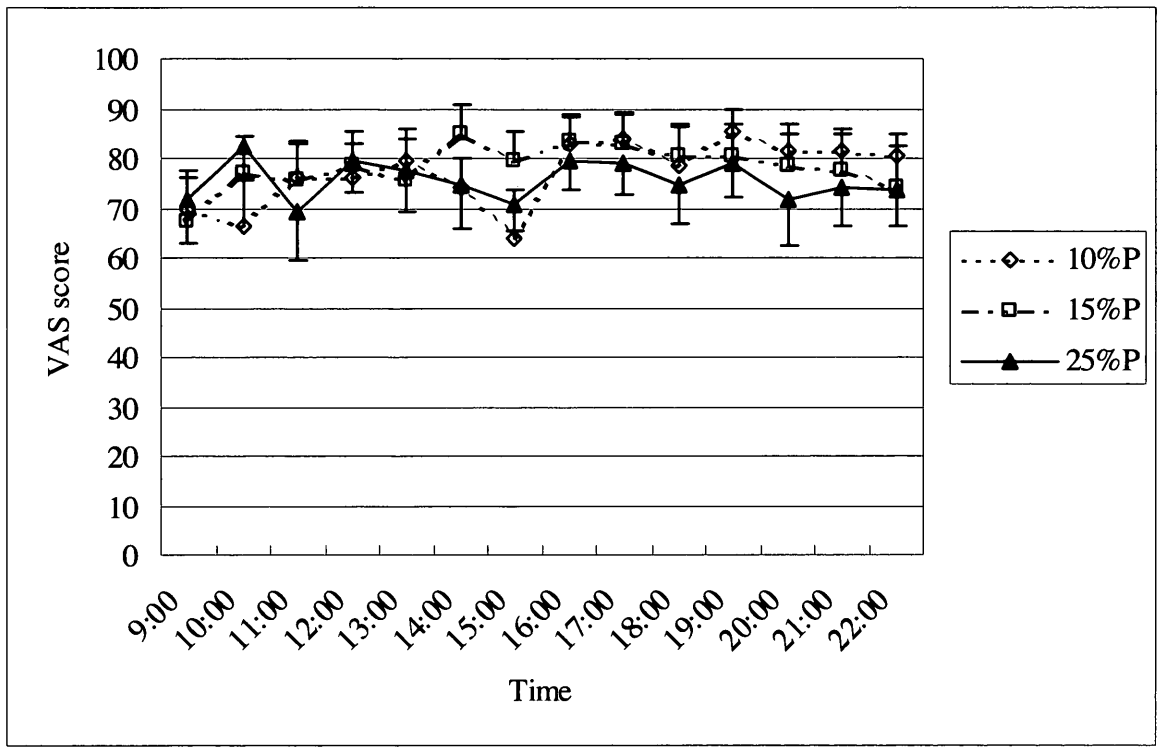



**Figure 4.3.21 Mean ± SEM VAS score 'How irritable are you?'**

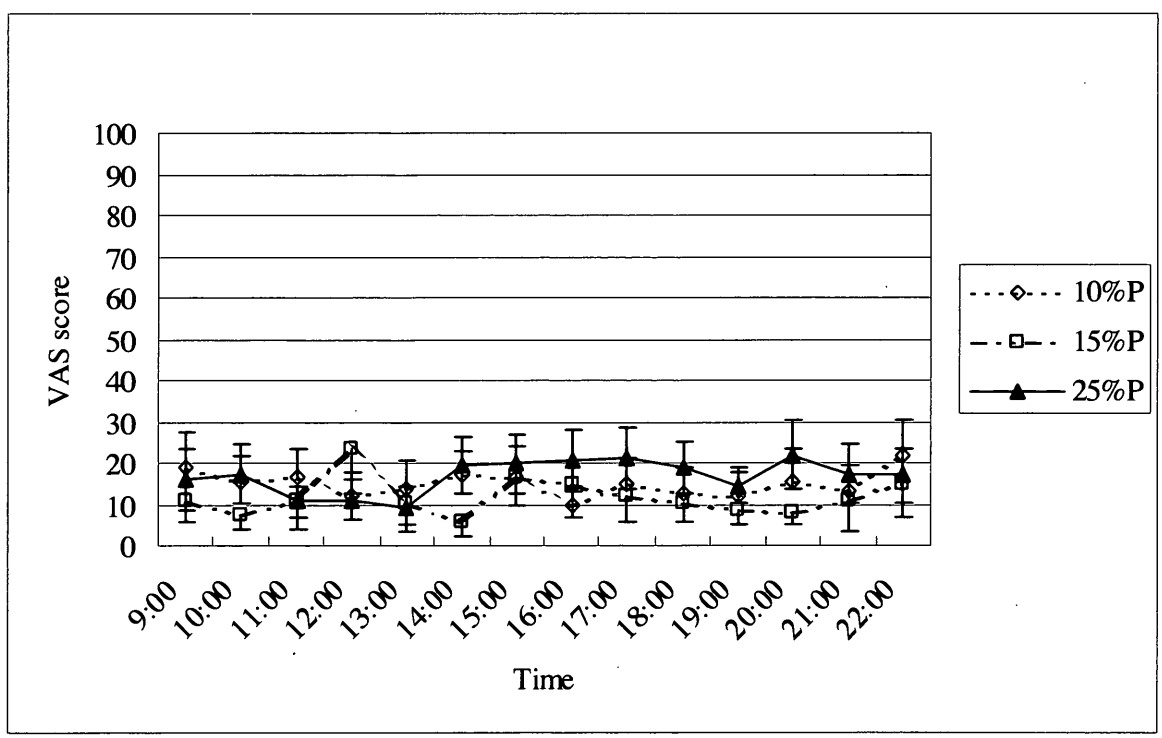


**Figure 4.3.22 Mean ± SEM VAS score 'How depressed are you?'**

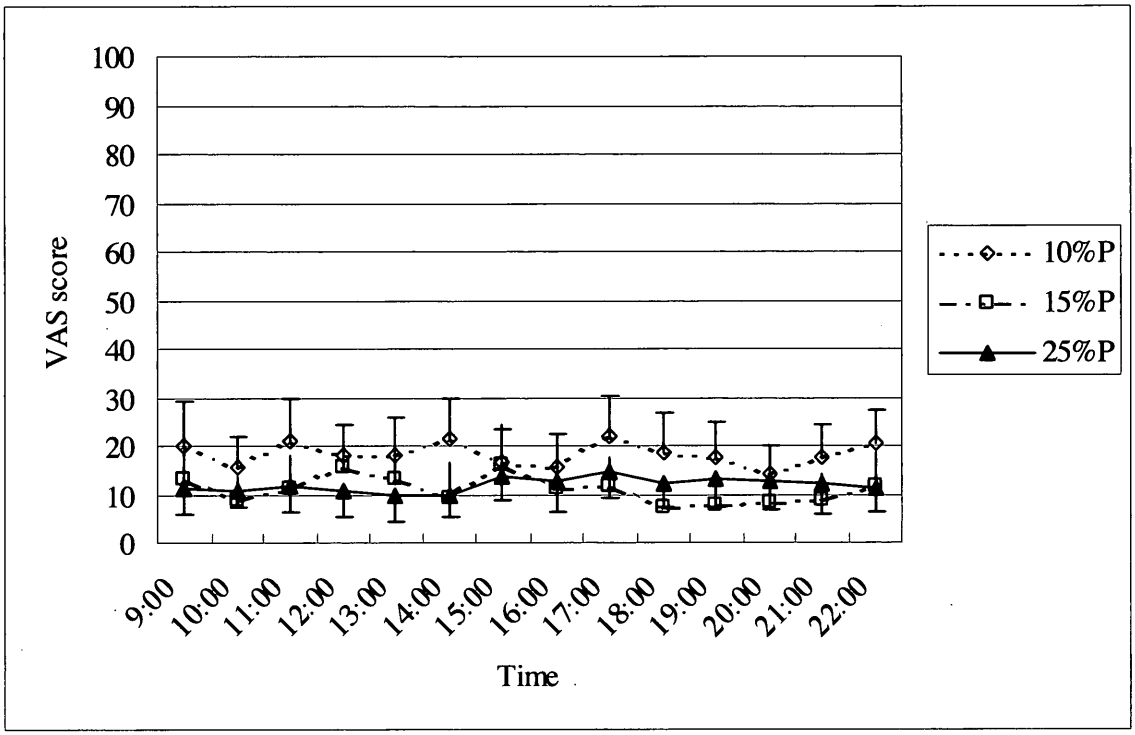



**Figure 4.3.23 Mean ± SEM VAS score 'How mentally alert are you?**

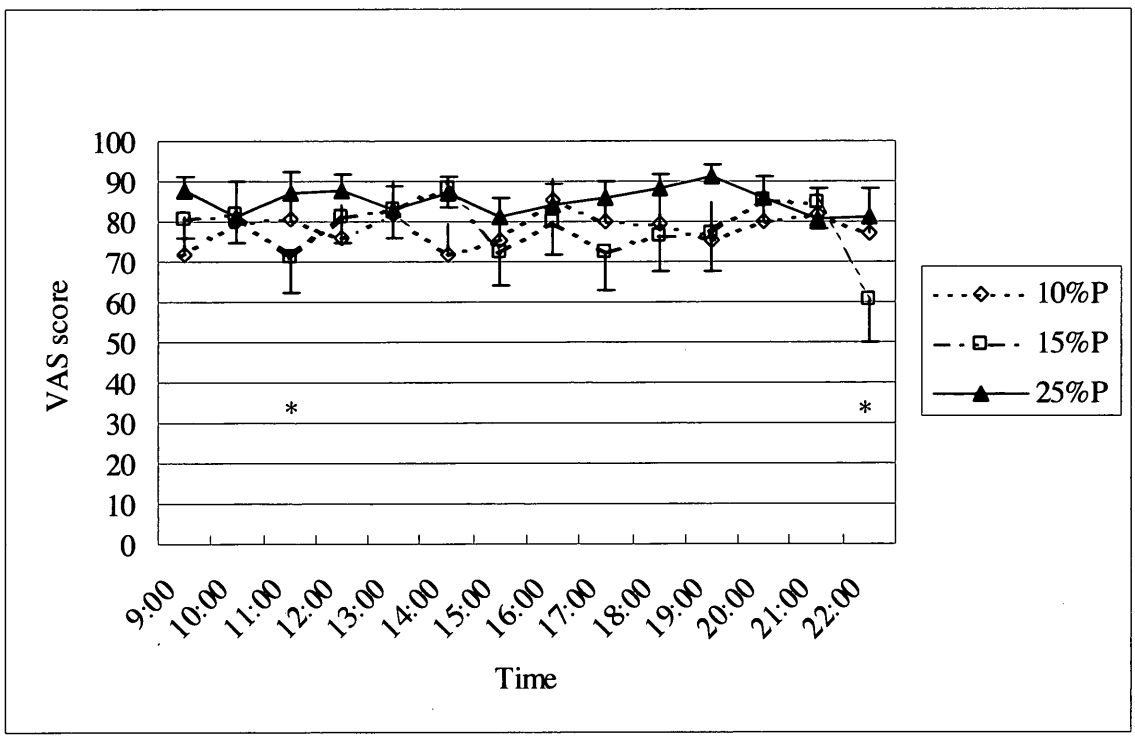


**Table 4.3.5 Mean ± SEM AUC for self-reported scores of hunger and satiety as assessed by VAS**

Vas detailed in page 248 and 4.3.3.3

| VAS | 10%P diet AUC score.h ± SEM | 15%P diet AUC score.h ± SEM | 25%P diet AUC score.h ± SEM | p value |
|---|---|---|---|---|
| **Hunger** | 302 ± 49 | 347 ±49 | 302 ± 49 | 0.14 |
| **Full** | 810 ± 76 | 788 ± 76 | 871 ± 76 | 0.004* |
| **Desire** | 290 ± 49 | 302 ± 49 | 286 ± 49 | 0.87 |
| **How much** | 328 ± 49 | 346 ± 49 | 302 ± 49 | 0.21 |
| **Content** | 1006 ± 71 | 1020 ± 71 | 988 ± 71 | 0.70 |
| **Irritable** | 193 ± 70 | 156 ± 70 | 214 ± 70 | 0.59 |
| **Depressed** | 239 ± 73 | 144 ± 73 | 154 ± 73 | 0.30 |
| **Alert** | 1051 ± 69 | 1016 ± 69 | 1110 ± 69 | 0.34 |

*4.3.3.4 Body mass*

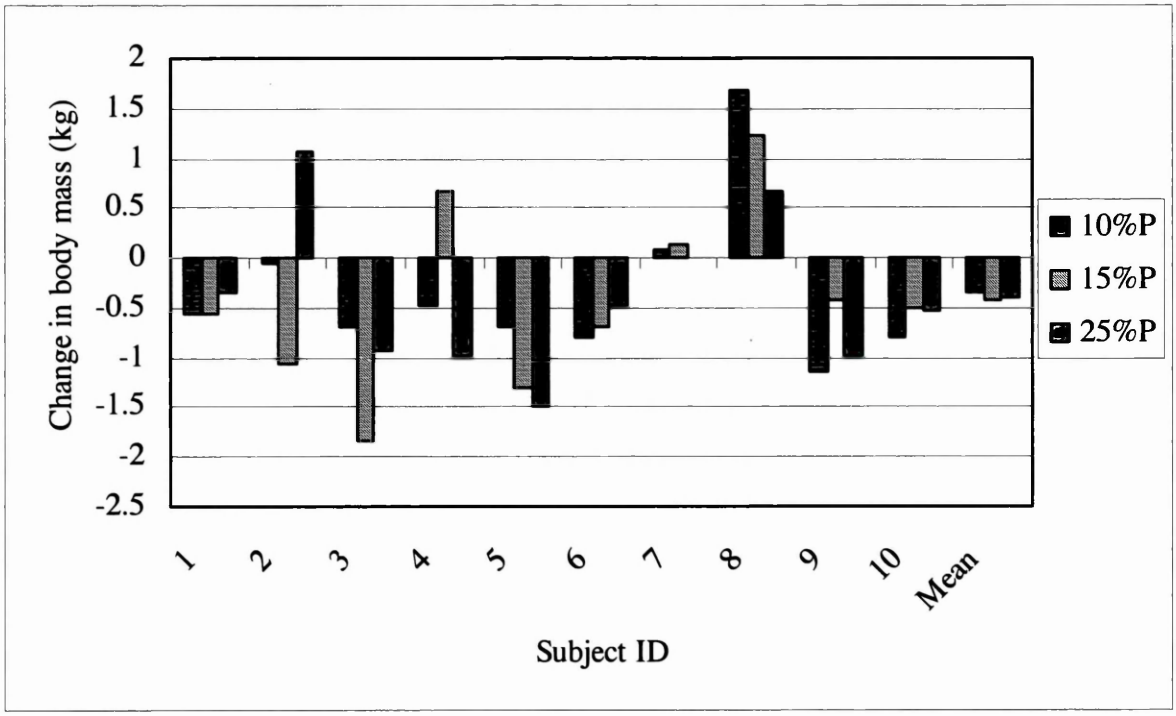
Subjects were weighed at the start and end of each study visit. There was no evidence of a difference in body mass on day 1 (p = 0.30) or day 5 (p = 0.31) of each study visit. The body mass difference between day 1 and day 5 was –0.3 kg (SD 0.8) during the 10%P diet, -0.4 kg (SD 0.9) during the 15%P diet, and –0.4 (SD 0.8) during the 25%P diet with no significant difference between each study diet (p = 0.94). Individual data are shown in Table 4.3.6 and Figure 4.3.24. The analysis was repeated excluding subject 8 whose food

consumption significantly exceeded energy requirements on all *ad libitum* eating days. Excluding this subject did not materially change the mean body mass on day 5 ($p = 0.06$) or the change in body mass ($p = 0.937$) for any of the study diets.

**Table 4.3.6 Body Mass (kg) of the study population – measured at baseline and at the end of each study visit.**

| Subject | 10% Protein | | | 15% Protein | | | 25% Protein | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 5 | Difference | Day 1 | Day 5 | Difference | Day 1 | Day 5 | Difference |
| 1 | 61.4 | 60.8 | -0.6 | 60.7 | 60.2 | -0.5 | 61.1 | 60.8 | -0.3 |
| 2 | 83.2 | 83.2 | 0.0 | 84.5 | 83.4 | -1.1 | 82.1 | 83.2 | 1.1 |
| 3 | 73.9 | 73.3 | -0.6 | 74.1 | 72.3 | -1.8 | 73.7 | 72.8 | -0.9 |
| 4 | 61.6 | 61.1 | -0.5 | 62.2 | 62.9 | 0.7 | 62.6 | 61.7 | -0.9 |
| 5 | 65.6 | 64.9 | -0.7 | 66.7 | 65.4 | -1.3 | 66.4 | 64.9 | -1.8 |
| 6 | 70.2 | 69.4 | -0.8 | 71.1 | 70.4 | -0.7 | 70.3 | 69.8 | -0.5 |
| 7 | 71.5 | 71.6 | 0.1 | 72.0 | 72.1 | 0.1 | 70.2 | 70.2 | 0.0 |
| 8 | 78.0 | 79.7 | 1.7 | 78.6 | 79.9 | 1.3 | 81.2 | 81.9 | 0.7 |
| 9 | 38.5 | 37.4 | -1.1 | 38.9 | 38.5 | -0.4 | 38.7 | 37.7 | -0.1 |
| 10 | 54.2 | 53.4 | -0.1 | 54.7 | 54.2 | -0.5 | 54.0 | 53.5 | -0.5 |
| Mean | 65.8 | 65.5 | -0.3 | 66.4 | 65.9 | -0.4 | 66.0 | 65.6 | -0.4 |
| Mean (excluding subject 8) | 64.5 | 63.9 | -0.6 | 65.0 | 64.4 | -0.6 | 64.3 | 63.8 | -0.5 |

**Figure 4.3.24 Change in body mass of subjects from day 1 to day 5 of each visit**




*4.3.3.5 Body Composition*

Measurement of body composition by air displacement plethysmography is presented in Table 4.3.7. Measurements of total body mass, fat mass and lean mass by DXA are also presented for comparison.

Mean fat mass did not differ between diets on day 1 or day 5 of each study visit but was significantly reduced on day 5 compared to day 1 on the 10%P diet ($p = 0.003$) and the 15%P diet ($p < 0.0001$). Mean fat-free mass was higher on day 5 on the 15%P diet than the 25%P diet ($p = 0.013$) and there was a significant increase in mean fat-free mass from day 1 to day 5 on the 15%P diet ($p = 0.045$).

There was no evidence of a difference between treatments in the change over five days in body mass (25%P –0.40 kg, SE 0.26, 15%P –0.34 kg, SE 0.26, 10%P –0.34kg, SE 0.26) (p = 0.96), fat mass (25%P –0.09 kg, SE 0.26, 15%P –0.82 kg, SE 0.26, 10%P –0.52 kg, SE 0.26) (p = 0.06) (Figure 4.3.25), or fat free mass (25%P 0.15 kg, SE 0.28, 15%P 0.48 kg, SE 0.27, 10%P –0.3 kg, SE 0.27) (p = 0.07) (Figure 4.3.26).

When body composition was measured by DXA, there was no evidence of a difference between diets in mean mass, fat mass or lean mass on day 1 or day 5 of each study diet. Fat mass was significantly lower on day 5 than day 1 on the 25%P diet (p = 0.0009) (Table 4.3.7).

There was no evidence of a difference between treatments in the change over five days in body mass (25%P –0.27 kg, SE 0.28, 15%P –0.27 kg, SE 0.28, 10%P –0.38kg, SE 0.28) (p = 0.87), or fat free mass (25%P 0.22 kg, SE 0.32, 15%P -0.36 kg, SE 0.32, 10%P – 0.16 kg, SE 0.32) (p = 0.21) (Figure 4.3.28) when measured by DXA. Fat mass fell significantly more on the 25%P diet than the 15%P diet (25%P –0.48 kg, SE 0.26, 15%P 0.071 kg, SE 0.13, 10%P –0.19 kg, SE 0.13) (p = 0.009) (Figure 4.3.27).

**Table 4.3.7 Mean (SEM) mass, fat mass, fat-free mass/lean mass measured by Bod Pod and DXA on day 1 and 5 of each diet**

Testing the change in mass between day 1 and day 5 (p*) and the difference between mass on day 1 and day 5 (p§).

| Diet | Total body mass (kg) | | | Fat mass (kg) | | | Fat-free mass (kg) | | |
|---|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 5 | p* | Day 1 | Day 5 | p* | Day 1 | Day 5 | p* |
| **Bod Pod** | | | | | | | | | |
| **10%P** | 65.8±4.1 | 65.5±4.1 | 0.17 | 16.1±2.0 | 15.6±2.0 | 0.03 | 49.7±3.5 | 49.9±3.6 | 0.34 |
| **15%P** | 66.2±4.1 | 65.8±4.1 | 0.23 | 16.6±2.0 | 15.8±2.0 | <0.001 | 49.6±3.6 | 50.0±3.6 | 0.04 |
| **25%P** | 66.1±4.2 | 65.7±4.2 | 0.11 | 16.5±2.2 | 16.4±2.2 | 0.8 | 49.6±3.5 | 49.3±3.6 | 0.43 |
| **p§** | 0.51 | 0.45 | | 0.44 | 0.17 | | 0.94 | 0.04 | |
| **DXA** | | | | | | | | | |
| 10%P | 65.9±4.2 | 65.5±4.2 | 0.10 | 16.6±2.3 | 16.4±2.3 | 0.14 | 46.4±3.4 | 46.3±3.4 | 0.58 |
| 15%P | 66.2±4.2 | 65.9±4.2 | 0.38 | 16.7±2.3 | 16.8±2.3 | 0.5 | 46.7±3.4 | 46.3±3.4 | 0.23 |
| 25%P | 66.2±4.2 | 65.9±4.2 | 0.34 | 17.1± 2.4 | 16.6±2.4 | 0.0009 | 46.3±3.3 | 46.5±3.3 | 0.55 |
| **p§** | 0.54 | 0.29 | | 0.25 | 0.27 | | 0.44 | 0.37 | |

**Figure 4.3.25 Change in fat mass from day 1 to day 5 of each study diet measured by Bod Pod for individual subjects**

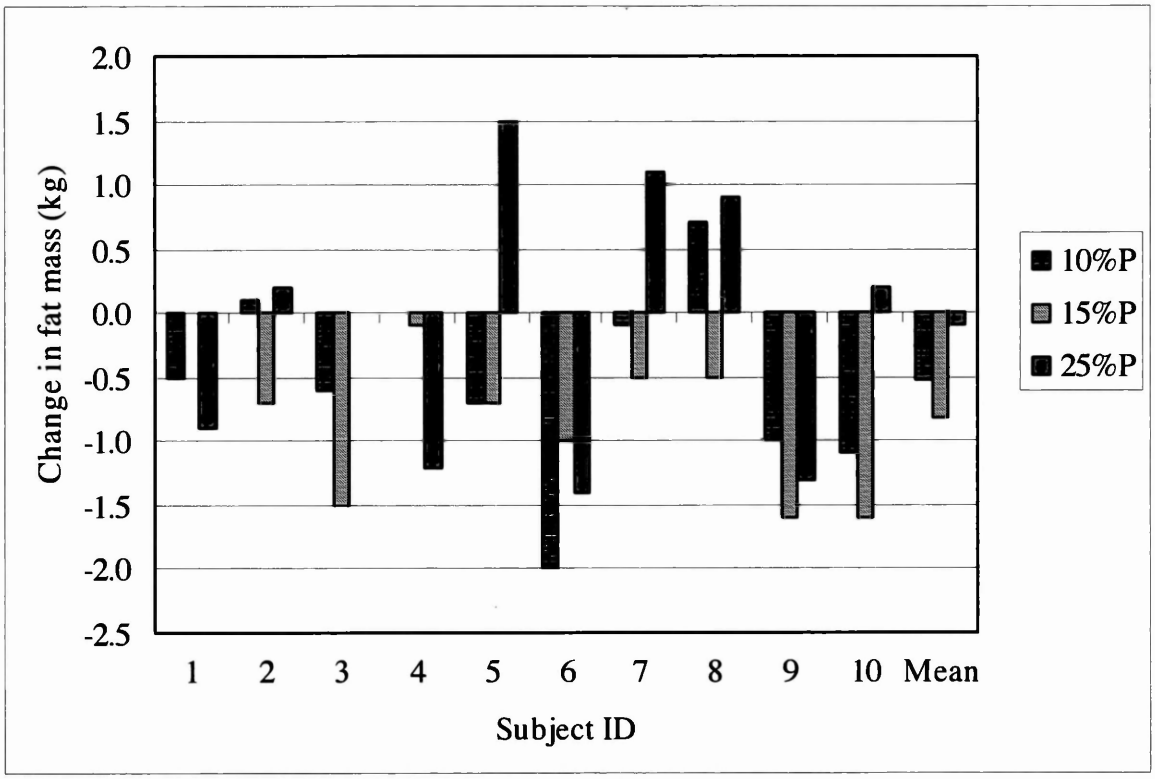



**Figure 4.3.26 Change in fat-free mass from day 1 to day 5 of each study diet measured by Bod Pod for individual subjects**

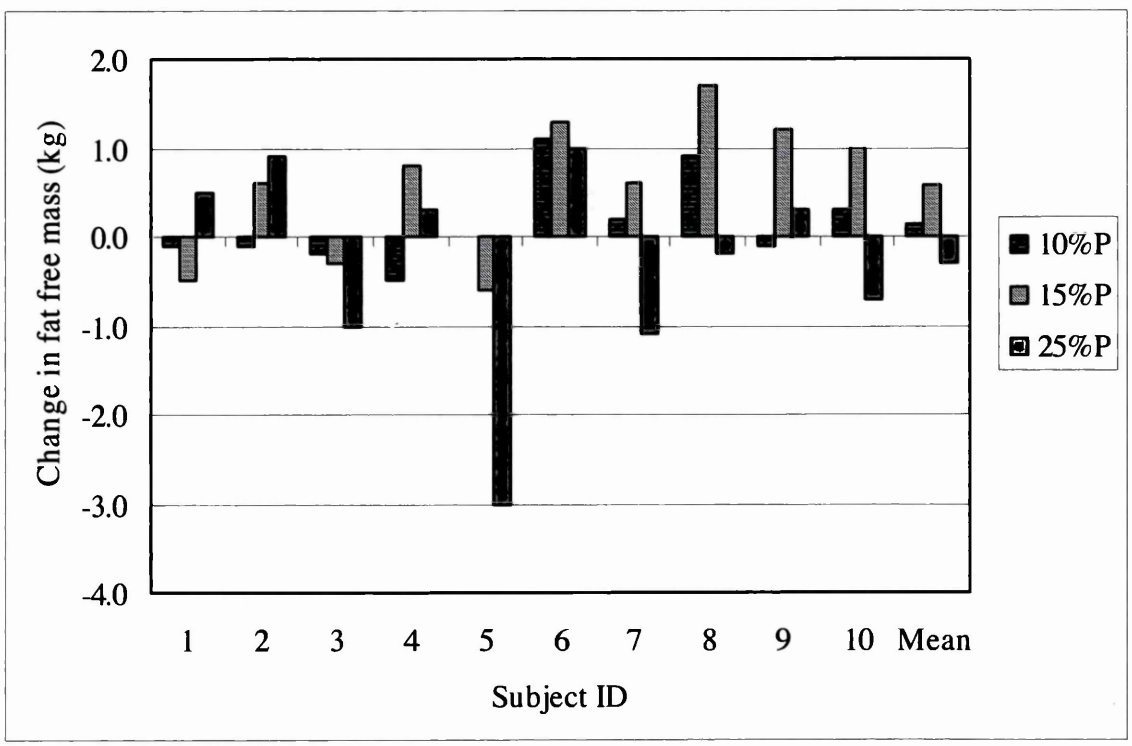


**Figure 4.3.27 Change in fat mass from day 1 to day 5 of each study diet measured by DXA for individual subjects**

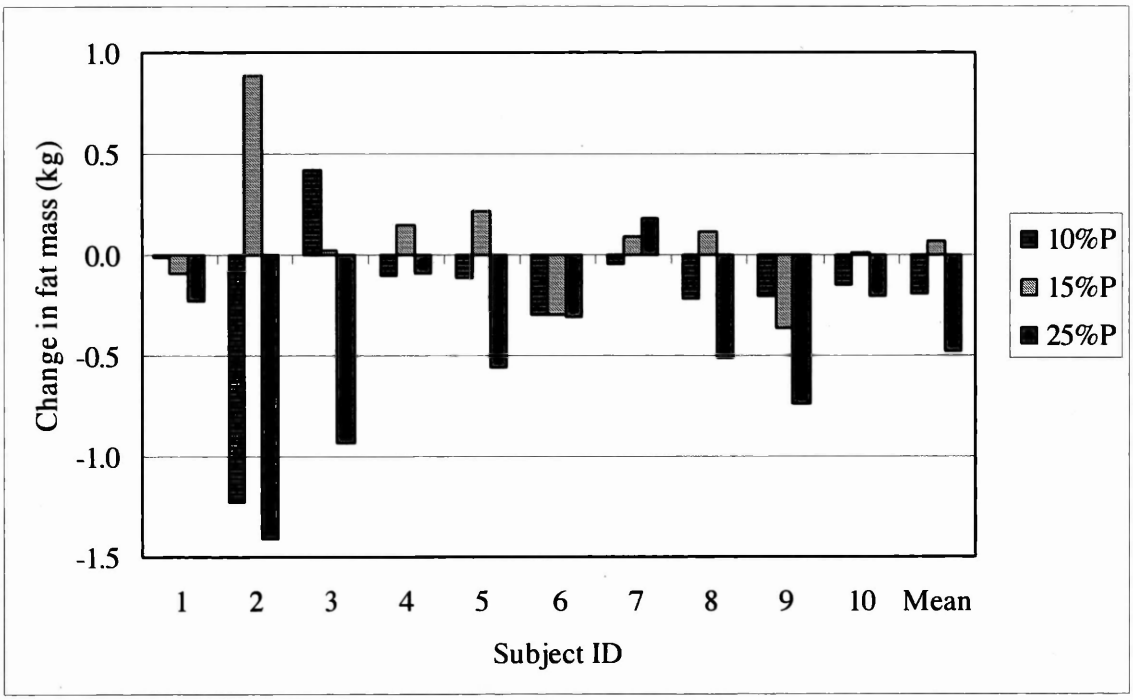
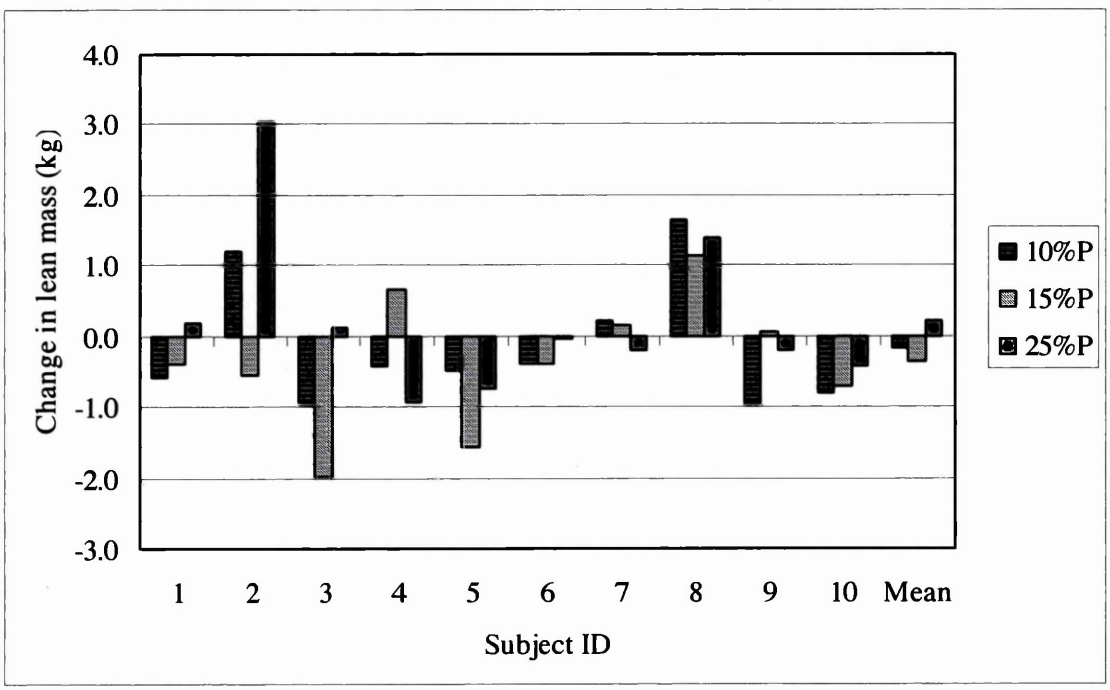
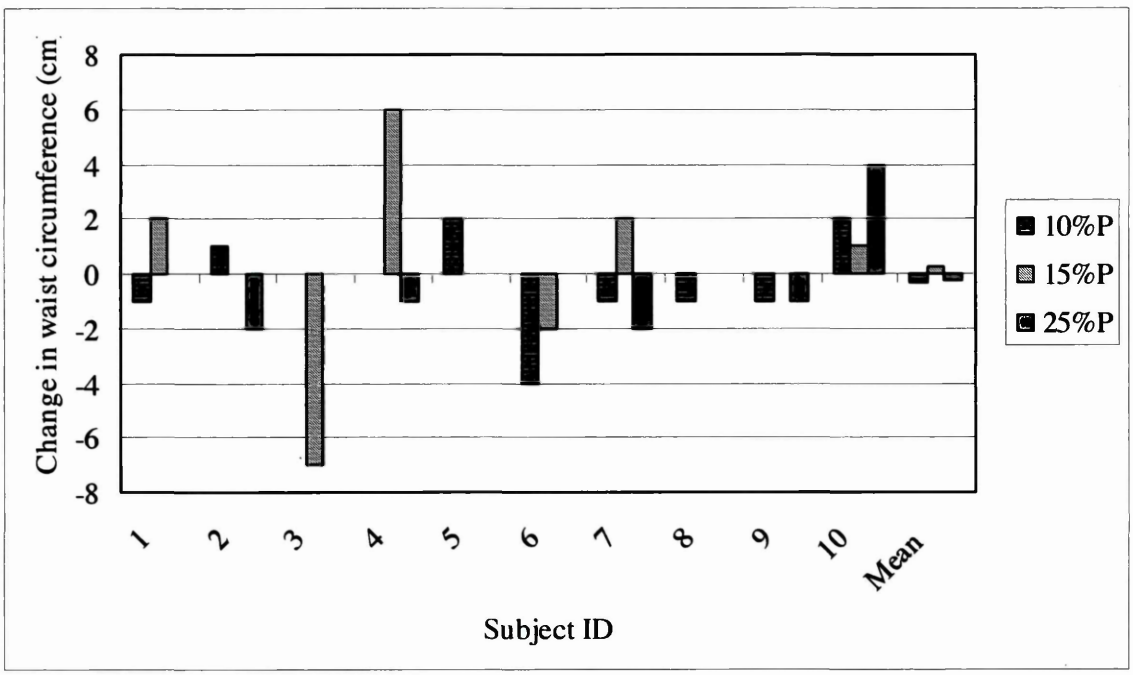



**Figure 4.3.28 Change in lean mass from day 1 to day 5 of each study diet measured by DXA for individual subjects**



There was no evidence of a difference in waist circumference between visits on day 1 (p = 0.34), day 5 (p = 0.76), or the change in waist circumference between day 1 and day 5 on each study visit (p = 0.88). Individual measurements are shown in Figure 4.3.29.

**Figure 4.3.29 Waist circumference difference of all subjects at each visit**




*4.3.3.6 Metabolic Risk*

Measurements of the change in systolic (SBP) and diastolic blood pressure (DBP) between day 1 and 5 of each study visit are shown in Figure 4.3.30 and Figure 4.3.31. There was no difference between the study visits for systolic blood pressure on day 1 (p = 0.15), day 5 (p = 0.97), or the change in systolic blood pressure between day 1 and day 5 (p = 0.46). Diastolic blood pressure was higher on day 1 of the 25%P visit (72.2 mmHg, SD 10.6) compared to day 1 on the 10%P visit (65.8 mmHg, SD 5.2) (p = 0.02). The day 1 DBP on the 15%P visit was 70 mmHg (SD 11.2). The difference in DBP was no longer significant when visit order was added to the model (p = 0.08). There was no difference between visits for the DBP on day 5 (p = 0.88), or between day 1 and day 5 (p = 0.30).

**Figure 4.3.30 Systolic blood pressure differences of all subjects at each visit**

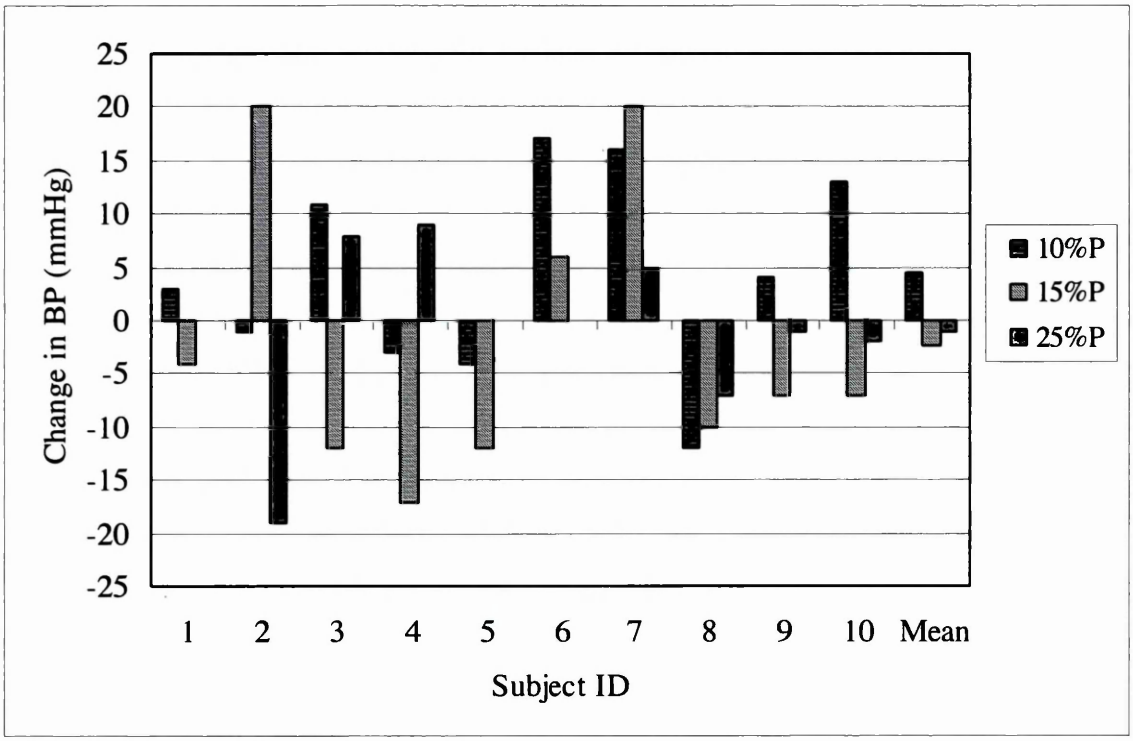
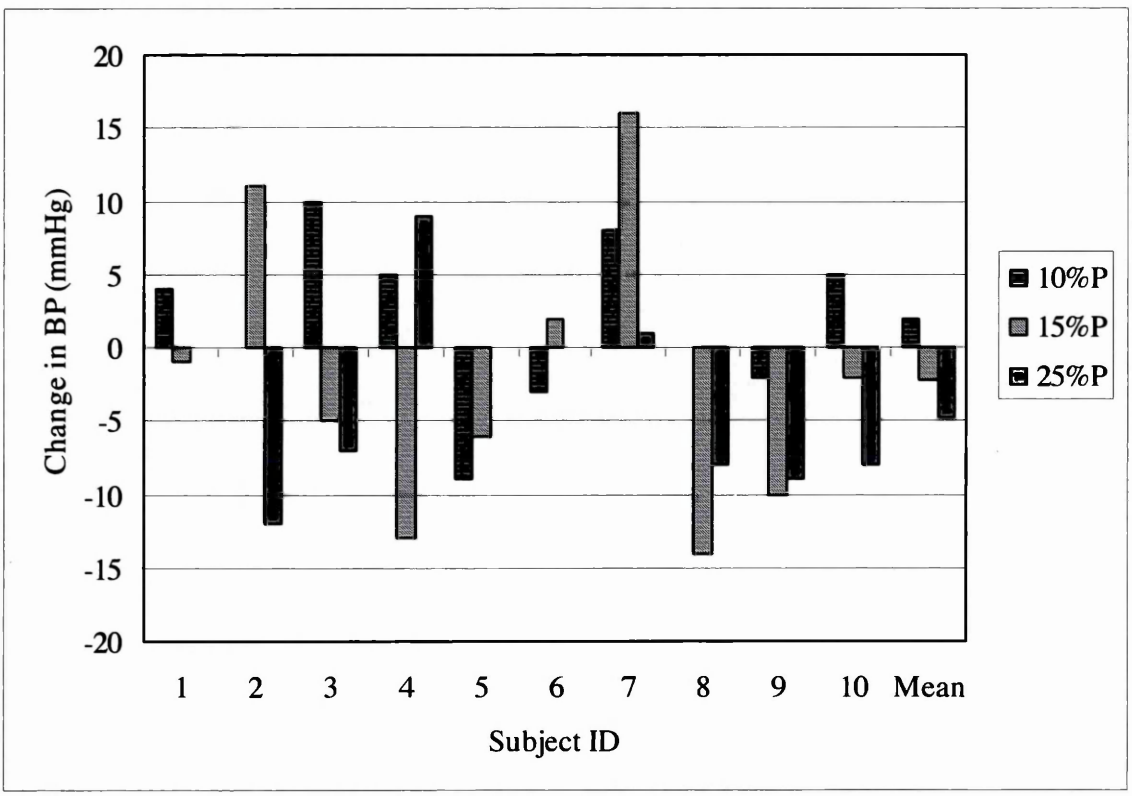



**Figure 4.3.31 Diastolic blood pressure differences of all subjects at each visit**

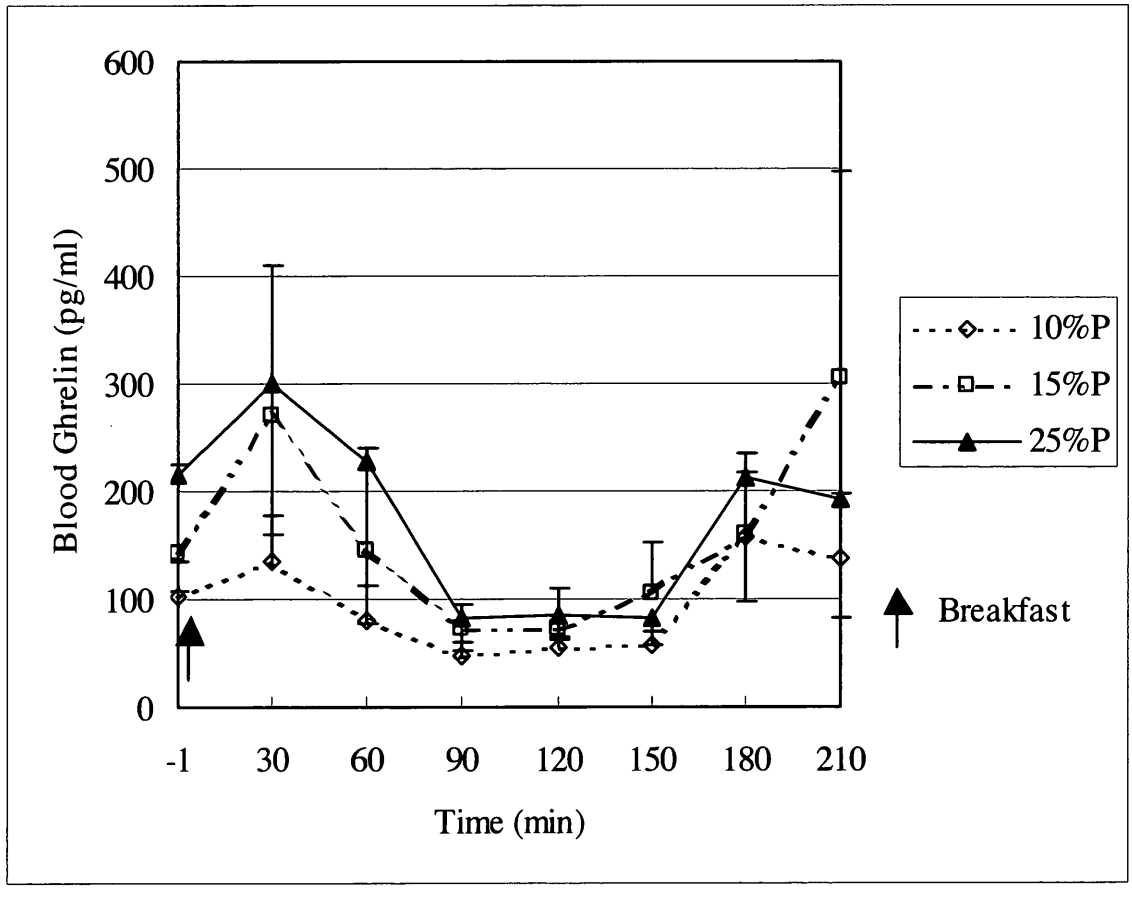


*4.3.3.7 Blood markers of satiety*

4.3.3.7.1 Ghrelin

After the fixed-energy breakfast there was no difference in subjects' blood ghrelin concentration between diets at baseline or at any other timepoint (Figure 4.3.32). There was a marked trend towards higher mean AUC as the percentage of protein increased: 10%P 18961 ± 12637 pg.min/ml, 15%P 28252 ± 12977 pg.min/ml, 25%P 35351 ± 12637 pg.min/ml; although this difference was not significantly different (p = 0.2). There was no evidence of a difference between the diets in ghrelin concentration or time of the maximum and minimum values.

**Figure 4.3.32 Mean ± SEM blood ghrelin after a fixed-energy breakfast eaten at time 0**

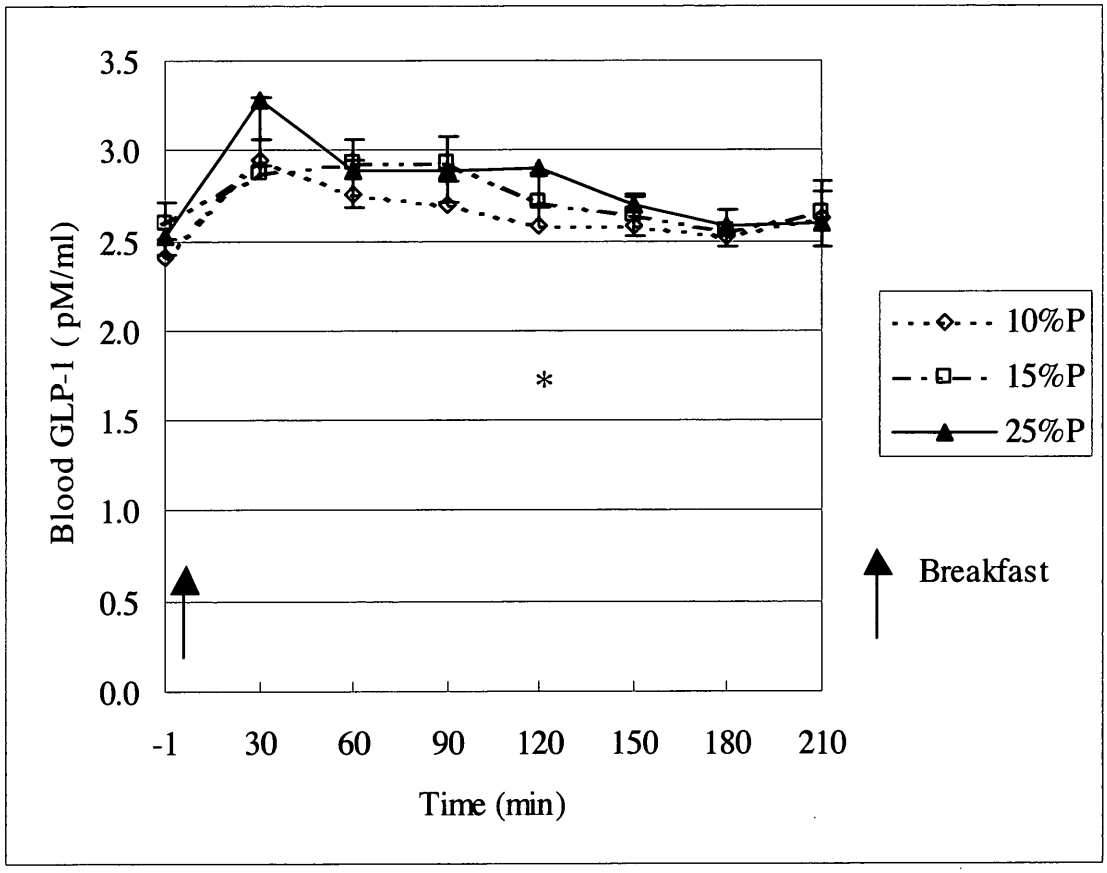


4.3.3.7.2 GLP-1

Subjects' blood GLP-1 did not differ between the diets at baseline, but at 120 min GLP-1 was significantly lower on the 10%P (2.6 ± 0.2 pM) diet compared to the 25%P diets (2.9 ± 0.2 pM), p = 0.018. (15%P 2.6 ± 0.2 pM). There was no difference between the diets at any other timepoint (Figure 4.3.33). The mean AUC increased as the percentage of protein increased, but the difference between the diets was not significant (p = 0.26). There was no evidence of a difference between the diets in concentration of GLP-1 or time of the maximum and minimum values.

**Figure 4.3.33 Mean ± SEM blood GLP-1 after a fixed-energy breakfast eaten at time 0**

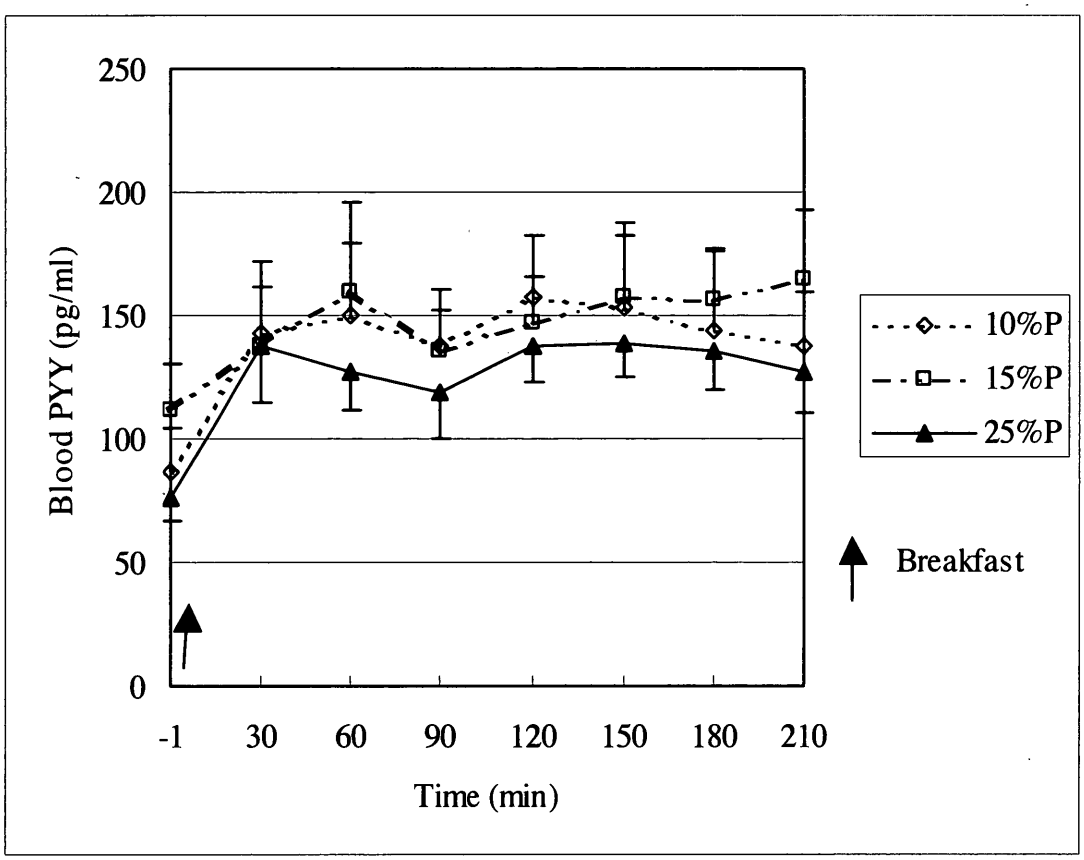
* indicates significant difference between the 10%P and 25%P diets



4.3.3.7.3 PYY

There was a difference in total PYY concentration at baseline between the 10%P (86 ± 17 pg/ml) and the 15%P (111 ± 18 pg/ml) and 25%P (76 ± 18 pg/ml) diets (p = 0.02). There was no difference at any other timepoint after the fixed-energy meal (Figure 4.3.34). The mean AUC did not differ between the diets (p = 0.28). There was a difference in the minimum concentration between the diets, corresponding to the difference in concentration at baseline. The time and concentration of the maximum PYY values were not different between the diets.

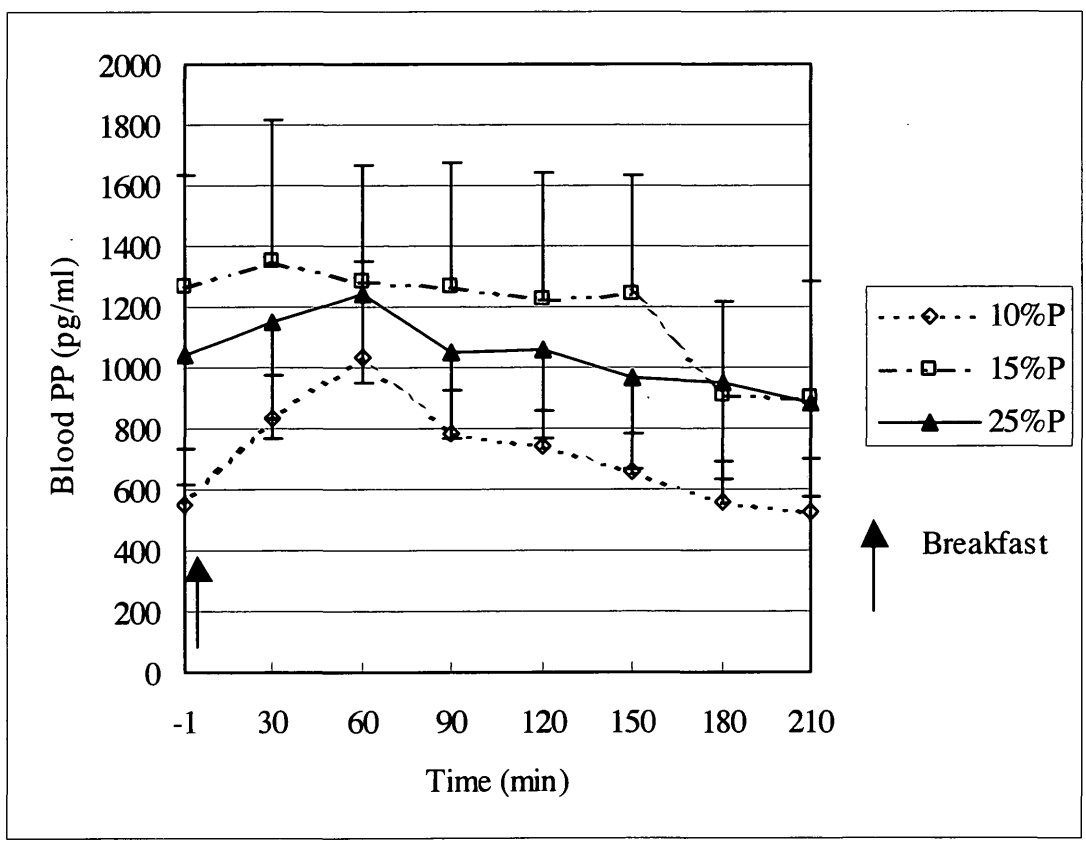
**Figure 4.3.34 Mean ± SEM blood PYY after a fixed-energy breakfast eaten at time 0**



4.3.3.7.4 PP

There was no difference in subjects' PP concentration between diets at baseline or at any timepoint after the fixed-energy meal (Figure 4.3.35). Mean AUC, lowest after the 10%P meal (153281 ± 38369 pg.min/ml), was significantly different to the 15%P meal (245269 ± 38859 pg.min/ml) and the 25%P meal (219920 ± 38369 pg.min/ml) ($p < 0.0001$). Similarly the maximum concentration of PP was lowest during the 10%P diet (1274 ± 200 pg/ml) compared to the 15%P (1637 ± 203 pg/ml) and 25%P (1567 ± 200 pg/ml) ($p = 0.018$). The minimum concentration was during the 10%P diet (334 ± 194 pg/ml) which was significantly lower than during the 15%P (697 ± 196 pg/ml) and the 25%P diets (656 ± 194 pg/ml) ($p < 0.0001$). The mean time to the minimum concentration was shorter during the 10%P diet (90 ± 24 min) than the 15%P diet (125 ± 24 min) ($p = 0.003$). The minimum concentration of PP during the 25%P diet was at 97 ± 24 min.

**Figure 4.3.35 Mean ± SEM blood PP after a fixed-energy breakfast eaten at time 0**



### *4.3.3.8 Blood markers of metabolic risk*

#### 4.3.3.8.1 Glucose

Figure 4.3.36 shows the change in individual fasting blood glucose between study day 1 and study day 4 for each diet. Fasting glucose concentration did not change during the study visits or differ between diets (mean 5.3 mmol/l, SD 0.4).

After the fixed-energy breakfast on day 4 there was no difference in glucose curves at baseline or at any other timepoint (Figure 4.3.37). The AUC did not differ between the diets ($p = 0.59$) and there was no evidence of a difference in the time or value of the maximum and minimum points.

**Figure 4.3.36 Change in fasting blood glucose between day 1 and day 4**

Excluding subject 9, from whom blood samples were not able to be taken

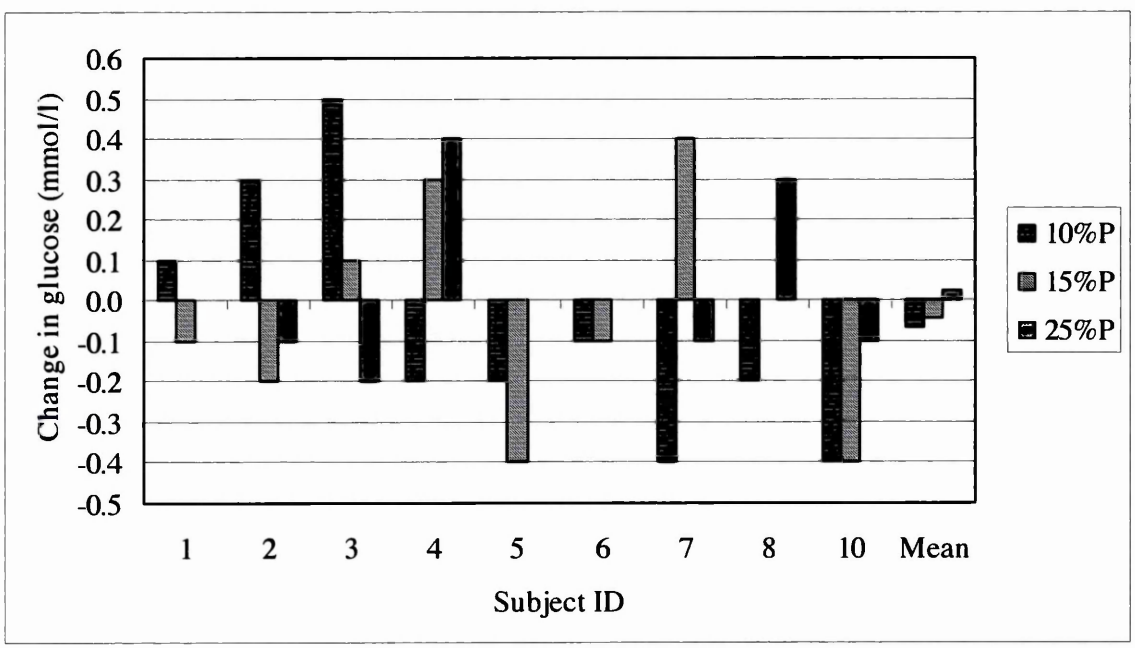
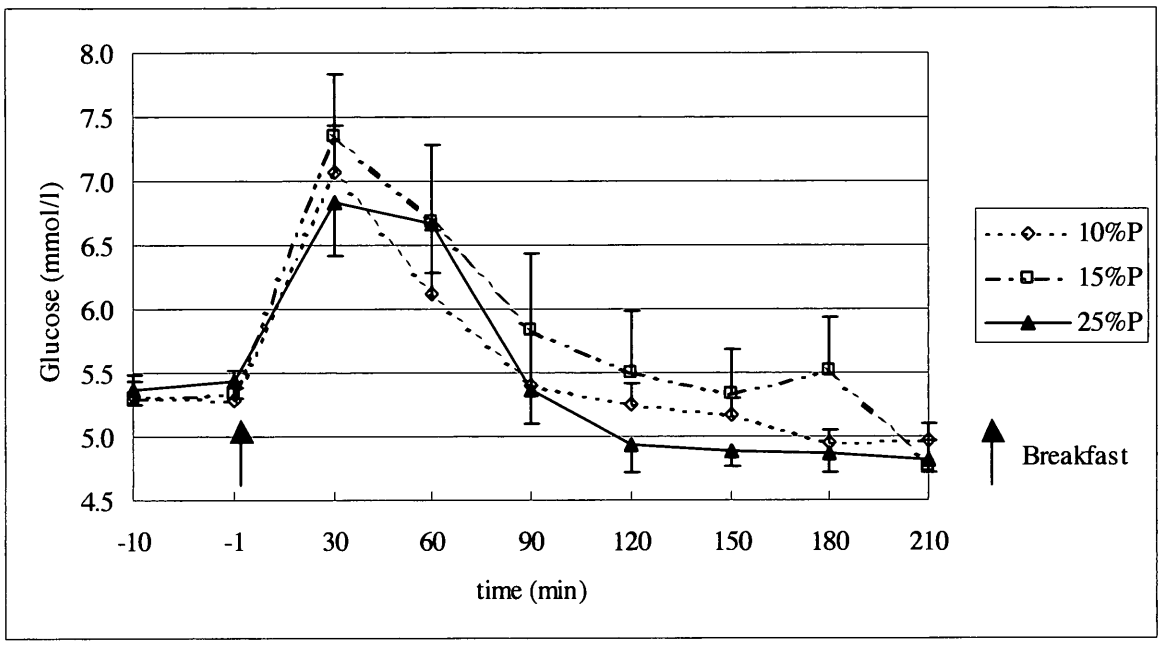


**Figure 4.3.37 Mean blood glucose after a fixed-energy breakfast (SEM)**




4.3.3.8.2 Continuous glucose monitoring

Interstitial blood glucose concentrations measured by the continuous glucose monitor were analysed during the final *ad libitum*-eating day and during the fixed-energy intake day spent in the calorimeter on day 4 of the study visit.

4.3.3.8.3 *Ad libitum*- eating day

Mean results for the *ad libitum* day are presented in Table 4.3.8 and Figure 4.3.38. Glucose concentrations were analysed for the whole 24 h period and the periods from waking to before breakfast (0700 – 0900), before breakfast to before lunch (0900 – 1230), before lunch to before dinner (1230 – 1730), before dinner to bed time (1730 – 2330), bedtime to waking (2330 – 0700). The time periods were selected to include the relevant meal for each subject within the time window. However, as subjects could elect to eat at any stage throughout the day, meals were consumed at different times within

these time periods and extra snacks may have additionally been consumed. The afternoon walk at 1500 h is marked on Figure 4.3.38.

The mean glucose concentration and AUC were significantly lower during the 25%P diet than the 10%P and 15%P diets for the entire 24 h *ad libitum* day and in all time periods, except between waking and breakfast. There was no evidence of a difference in the mean AUC between the 10%P and 15%P diets at any time throughout the day.

There was no difference in mean glucose concentration at 0700 h, 0900 h, 1230 h, or 1730 h. At 2330 h mean glucose concentration was significantly lower during the 25%P diet than the 10%P or 15%P ($p = 0.03$). At 0700 the following morning the mean glucose concentration was significantly lower during the 25%P diet than the 15%P ($p = 0.03$).

The maximum glucose concentration was lower between breakfast and lunch during the 25%P diet (6.51 mmol/l ± 0.46) than the 10%P (7.78 mmol/l ± 0.47) ($p = 0.013$) and 15%P diets (7.07 mmol/l ± 0.47) ($p = 0.045$), and between lunch and dinner the concentration was lower during the 25%P diet (6.45 mmol/l ± 0.42) than the 10%P (7.84 mmol/l ± 0.40) ($P = 0.001$) and 15%P diets (7.29 mmol/l ± 0.41) ($p = 0.044$). There was no difference during other time periods or for the whole 24 h period.

The minimum glucose concentration was lower between breakfast and lunch during the 25%P (4.67 mmol/l ± 0.19) ($p = 0.004$) and 10%P diets (4.88 mmol/l ± 0.19) ($p = 0.03$) than the 15%P diet (5.33 mmol/l ± 0.19), and was lower overnight during the 25%P diet (4.65 mmol/l ± 0.25) than the 15%P diet (5.47 mmol/l ± 0.26) ($p = 0.007$). The glucose concentration during the 10%P diet was 5.06 mmol/l ± 0.25 and did not differ from the other two diets.

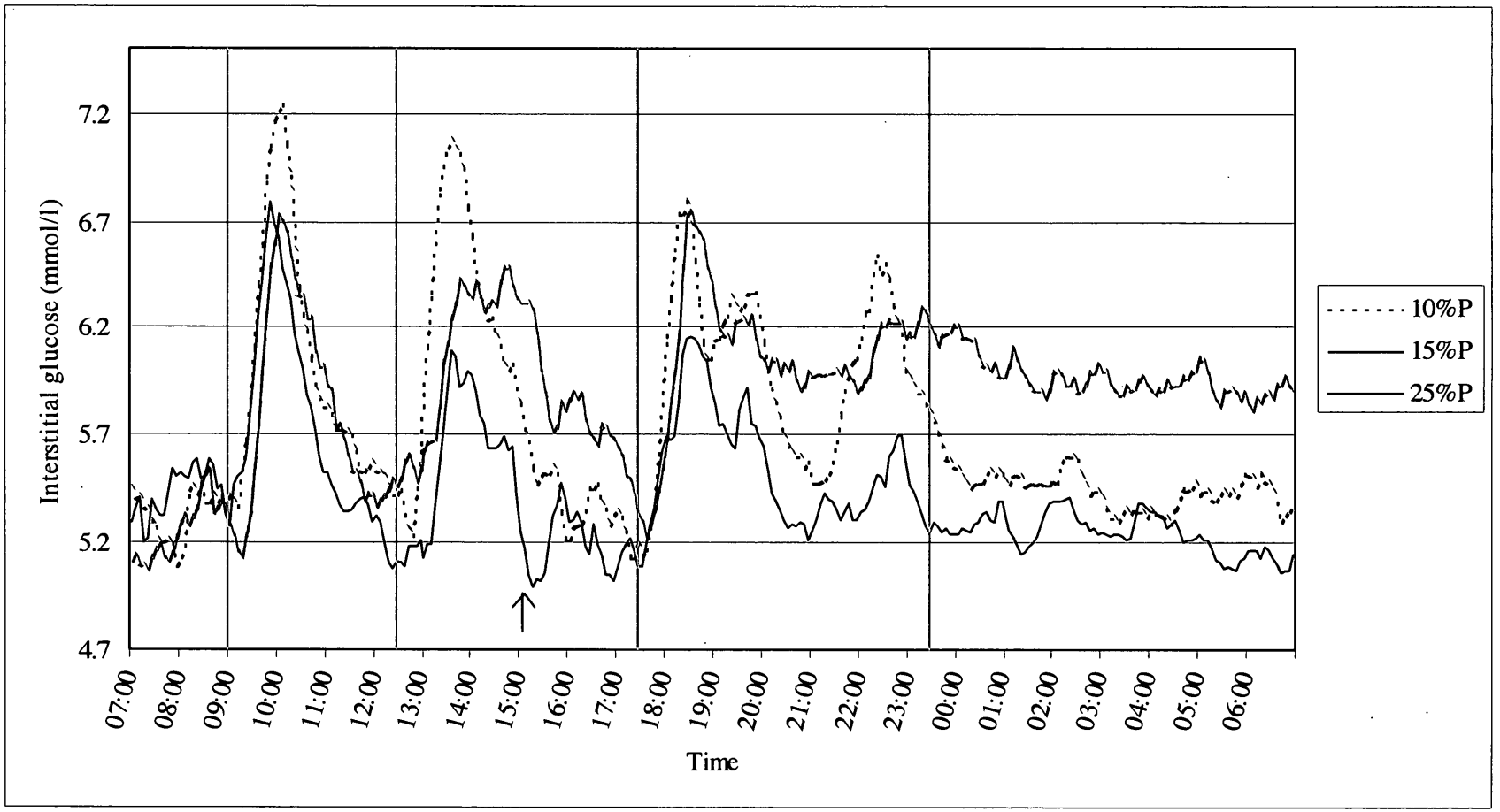
**Table 4.3.8 Mean ± SEM interstitial glucose concentration and AUC during the 24 h *ad libitum* period and specific time periods throughout the day for all subjects**

| Time | 10%P | 15%P | 25%P | p |
|---|---|---|---|---|
| **Mean interstitial glucose (mmol/l)** | | | | |
| **Mean over 24 h** | 5.90 ± 0.2 | 5.89 ± 0.2 | 5.44 ± 0.2 | <0.0001*§ |
| **0700 - 0900** | 5.47 ± 0.2 | 5.29 ± 0.2 | 5.44 ± 0.2 | 0.01§# |
| **0900 - 1230** | 6.06 ± 0.2 | 6.01 ± 0.2 | 5.46 ± 0.2 | <0.0001*§ |
| **1230 – 1730** | 5.94 ± 0.2 | 5.90 ± 0.2 | 5.38 ± 0.2 | <0.0001*§ |
| **1730 – 2330** | 6.22 ± 0.2 | 6.08 ± 0.2 | 5.55 ± 0.2 | <0.0001*§# |
| **2330 - 0700** | 5.65 ± 0.2 | 5.96 ± 0.2 | 5.24 ± 0.2 | <0.0001*§# |
| | | | | |
| **Mean AUC (mmol.min/l)** | | | | |
| **Mean over 24 h** | 8470 ± 337 | 8464 ± 347 | 7800 ± 337 | 0.04 *§ |
| **0700 - 0900** | 628 ± 30.9 | 606 ± 32.4 | 626 ± 30.9 | 0.8 |
| **0900 - 1230** | 1241 ± 50.2 | 1241 ± 50.2 | 1120 ± 48.2 | 0.03*§ |
| **1230 – 1730** | 1755 ± 72.6 | 1741 ± 75.2 | 1574 ± 75.2 | 0.02*§ |
| **1730 – 2330** | 2208 ± 105.9 | 2157 ± 110.1 | 1969.7 ± 105.9 | 0.03* |
| **2330 - 0700** | 2513 ± 113.9 | 2652 ± 118.2 | 2333 ± 113.9 | 0.03§ |

§ 25%P diet different from 15%P, * 25%P diet different from 10%P, # 15%P diet different from 10%P

**Figure 4.3.38 Mean 24-hour interstitial glucose profiles of all subjects during the *ad libitum* day of each diet.**

Horizontal lines indicate where the day was separated into specific time periods as detailed in 4.3.3.8.3.




↑ Exercise

4.3.3.8.4 Fixed-energy intake day

The results for the fixed-energy intake day are shown in Table 4.3.9 and Figure 4.3.39. As all activities and eating episodes occurred at the same time for all subjects, the day was separated into time periods dependent on meal times. Meals were eaten at 0930 h, 1330 h and 1830 h and so the day was divided into waking to before breakfast (0700 – 0930), before breakfast to before lunch (0930 – 1330), before lunch to before dinner (1330 – 1830), before dinner to bed time (1830 – 2330), bedtime to waking (2330 – 0700). No snacks were provided and only decaffeinated drinks were served. Two periods of standardised exercise were performed at 1500 h and 2000 h marked on Figure 4.3.39.

There was no difference in mean glucose concentration at 0700, 1330, 1830, 2330, or 0700 the following morning. Mean glucose was significantly higher at 0930 during the 15%P diet (5.77 ± 0.23) than the 10%P (5.25 ± 0.23) and 25%P diets (5.18 ± 0.22) ($p = 0.01$).

Mean glucose concentration was significantly lower during the 25%P diet than the 10%P and 15%P during all time periods and for the entire 24 h period. During the 15%P diet mean glucose concentration was lower than the 10%P diet for the entire 24 h period and from lunch to dinner, dinner to bedtime, and overnight Table 4.3.9. The AUC was lower during the 25%P diet than the 15%P or 10%P between breakfast and lunch, and during the 25%P and 15%P diets compared to the 10%P diets between dinner and bedtime. There was no difference in the 24 h AUC.

The maximum glucose concentration was lower for the whole 24 h period during the 25%P diet (6.96 ± 0.56) than the 15%P (7.49 ± 0.58) ($p < 0.0001$) and 10%P diets (9.32 ± 0.62) ($p < 0.0001$). The 15%P diet was lower than the 10%P diet ($p = 0.007$). The maximum glucose concentration was also lower between breakfast and lunch during the 25%P diet (6.55 ± 0.47) than the 15%P diet (7.14 ± 0.47) which was lower than the 10%P diet (8.05 ± 0.49) ($p = 0.02$), between lunch and dinner during the 25%P diet (6.06 ± 0.37) and the 15%P diet (6.47 ± 0.39) than the 10%P diet (7.75 ± 0.41) ($p = 0.001$), from dinner to bedtime during the 25%P (6.79 ± 0.60) and 15%P (6.88 ± 0.62) than the 10%P diet (9.28 ± 0.70) ($p = 0.002$). There were no differences between diets in the period between waking and breakfast, or overnight.

The time to maximum glucose concentration was later during the 25%P diet than the 10% and 15%P diets ($p = 0.0001$) between lunch and dinner and earlier during the 25%P diet and the 15%P diets than the 10%P diets ($p = 0.04$) overnight. There were no differences at other periods throughout the day.

The minimum glucose concentration was lower between waking and breakfast during the 25%P (4.97 ± 0.22) and 10%P (5.14 ± 0.24) than the 15%P diets (5.58 ± 0.23) ($p = 0.007$), and between breakfast and lunch during the 25%P diet (4.69 ± 0.19) than 15%P diet (5.23 ± 0.19) ($p = 0.03$). The 10%P diet did not differ from the other two (4.8 ± 0.2). There was no difference in the time to minimum concentration at any period throughout the day.

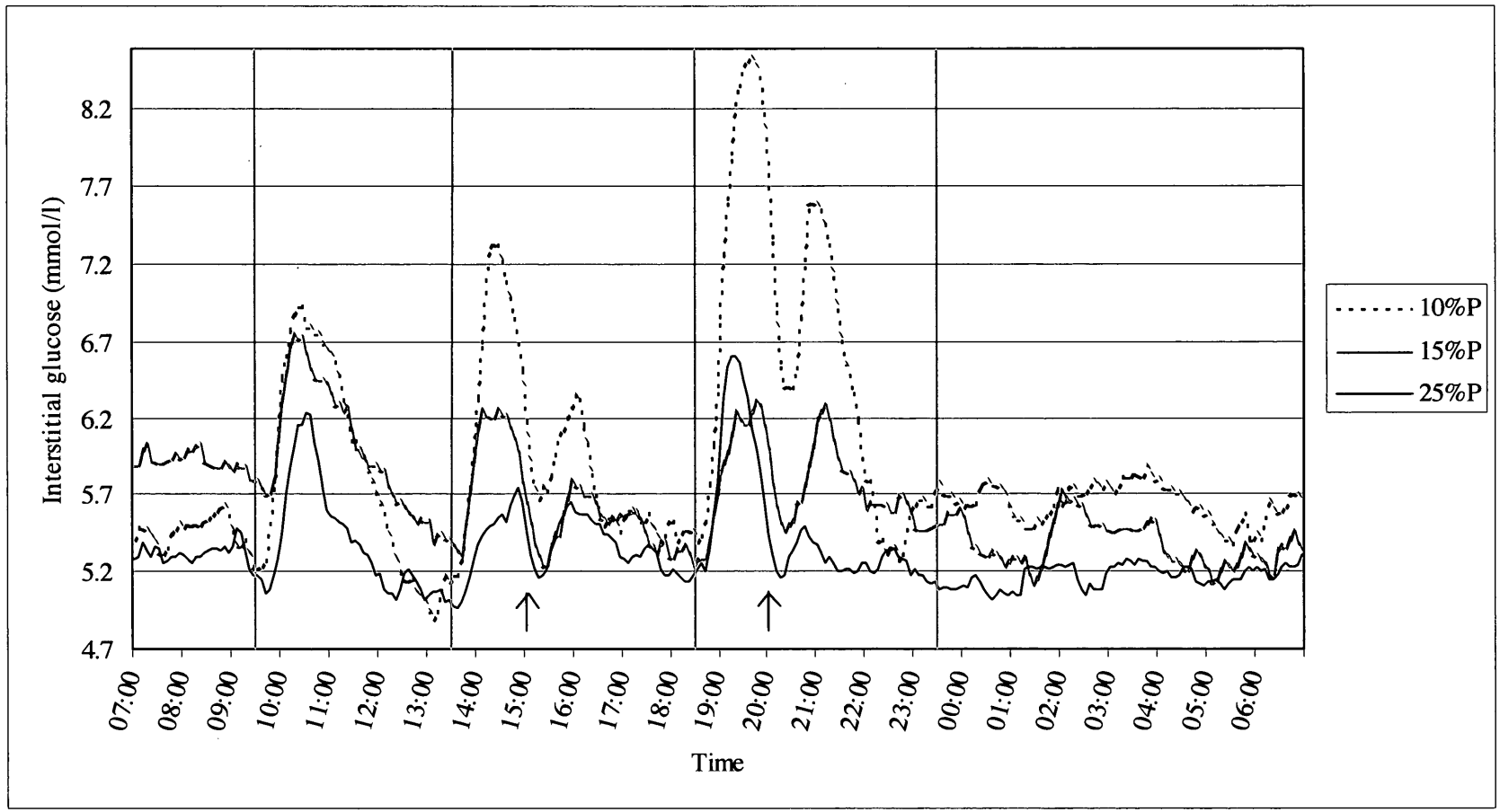
**Table 4.3.9 Mean ± SEM interstitial glucose concentration and AUC at specific time periods for all subjects eating the fixed-energy meals over 24 hours**

| Time | 10%P | 15%P | 25%P | p |
|---|---|---|---|---|
| **Mean interstitial glucose (mmol/l)** | | | | |
| **Mean over 24 h** | 5.95 ± 0.16 | 5.66 ± 0.16 | 5.31 ± 0.16 | <0.0001*§# |
| **0700 - 0930** | 5.52 ± 0.21 | 5.91 ± 0.21 | 5.32 ± 0.21 | <0.0001*§# |
| **0930 - 1330** | 5.89 ± 0.17 | 5.90 ± 0.17 | 5.39 ± 0.17 | <0.0001*§ |
| **1330 – 1830** | 5.97 ± 0.17 | 5.63 ± 0.17 | 5.36 ± 0.17 | <0.0001*§# |
| **1830 – 2330** | 6.69 ± 0.21 | 5.79 ± 0.21 | 5.48 ± 0.21 | <0.0001*§# |
| **2330 - 0700** | 5.70 ± 0.19 | 5.41 ± 0.19 | 5.14 ± 0.19 | <0.0001*§# |
| | | | | |
| **Mean AUC (mmol.min/l)** | | | | |
| **Mean over 24 h** | 8205 ± 447 | 8121 ± 424 | 7463 ± 404 | 0.27 |
| **0700 - 0930** | 804 ± 37 | 859 ± 36 | 772 ± 35 | 0.01§ |
| **0930 - 1330** | 1422 ± 50 | 1405 ± 48 | 1271 ± 48 | 0.007*§ |
| **1330 – 1830** | 1758 ± 78 | 1660 ± 74 | 1581 ± 71 | 0.13 |
| **1830 – 2330** | 2021 ± 102 | 1706 ± 91 | 1617 ± 87 | 0.002*# |
| **2330 - 0700** | 2510 ± 125 | 2377 ± 113 | 2318 ± 113 | 0.37 |

* 25%P diet different from 10%P, § 25%P diet different from 15%P, # 15%P diet different from 10%P

**Figure 4.3.39 Mean 24-hour interstitial glucose profiles during the fixed energy intake day of each diet.**

Horizontal lines indicate where the day was separated into specific time periods as detailed in **4.3.3.8.4**




↑ Exercise

The mean glucose concentrations and AUC for the entire 24 h period are presented in Table 4.3.10 for the *ad libitum* day and the fixed-energy day. The mean glucose concentration was lower during the fixed energy day for the 15%P and 25%P diets. There was no difference in mean AUC. The days differed by the number of eating episodes, with no snacks provided on the fixed-energy day, however energy intake did not differ significantly between the days (Figure 4.3.14).

**Table 4.3.10 Mean interstitial glucose and AUC over 24 h during the *ad libitum* and fixed-energy intake days**

| **Diet** | ***Ad libitum* day** | **Fixed-energy day** | **p** |
|---|---|---|---|
| Mean interstitial glucose (mmol/l) | | | |
| 10%P | 5.90 ± 0.2 | 5.95 ± 0.16 | 0.003 |
| 15%P | 5.89 ± 0.2 | 5.66 ± 0.16 | <0.0001 |
| 25%P | 5.44 ± 0.2 | 5.31 ± 0.16 | <0.0001 |
| Mean AUC (mmol.min/l) | | | |
| 10%P | 8470 ± 337 | 8205 ± 447 | 0.51 |
| 15%P | 8464 ± 347 | 8121 ± 424 | 0.16 |
| 25%P | 7800 ± 337 | 7463 ± 404 | 0.94 |

4.3.3.8.5 Insulin

Subjects' mean fasting blood insulin concentration was higher on day 4 than day 1 during each of the study diets (Figure 4.3.40), but there was no difference between diets in the change in insulin concentration during the study week (p = 0.28). The individual difference between day 1 and day 4 is shown in Figure 4.3.41. Fasting insulin concentrations did not differ when adjusted for day of the study week or diet.

Post-prandial insulin curves are shown in Figure 4.3.42. There was no difference in insulin concentration at baseline or at any other timepoint after the fixed-energy breakfast on day 4. The AUC did not differ between diets (p = 0.25), neither did the time and concentration of the maximum or minimum values.

**Figure 4.3.40 Mean (SEM) fasting blood insulin concentrations on days 1 and 4 of each study diet for all subjects**

| Diet | Insulin Concentration Day 1 mean±SEM | Insulin Concentration Day 4 mean±SEM | p for difference between day 1 and day 4 |
|---|---|---|---|
| 10%P | 27.9 ± 4.6 | 44.4 ± 4.6 | <0.0001 |
| 15%P | 27.9 ± 4.1 | 35.7 ± 4.1 | 0.03 |
| 25%P | 22.8 ± 4.3 | 38.2 ± 4.0 | 0.002 |

**Figure 4.3.41 Changes in blood insulin concentration between day 1 and day 4 of each study visit for each subject**

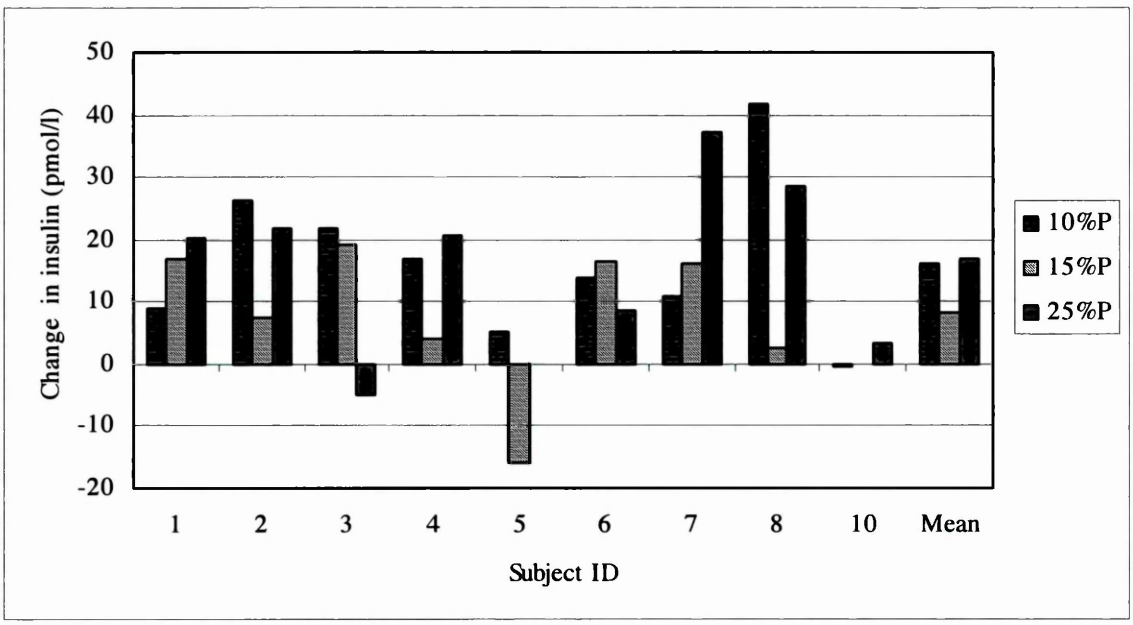
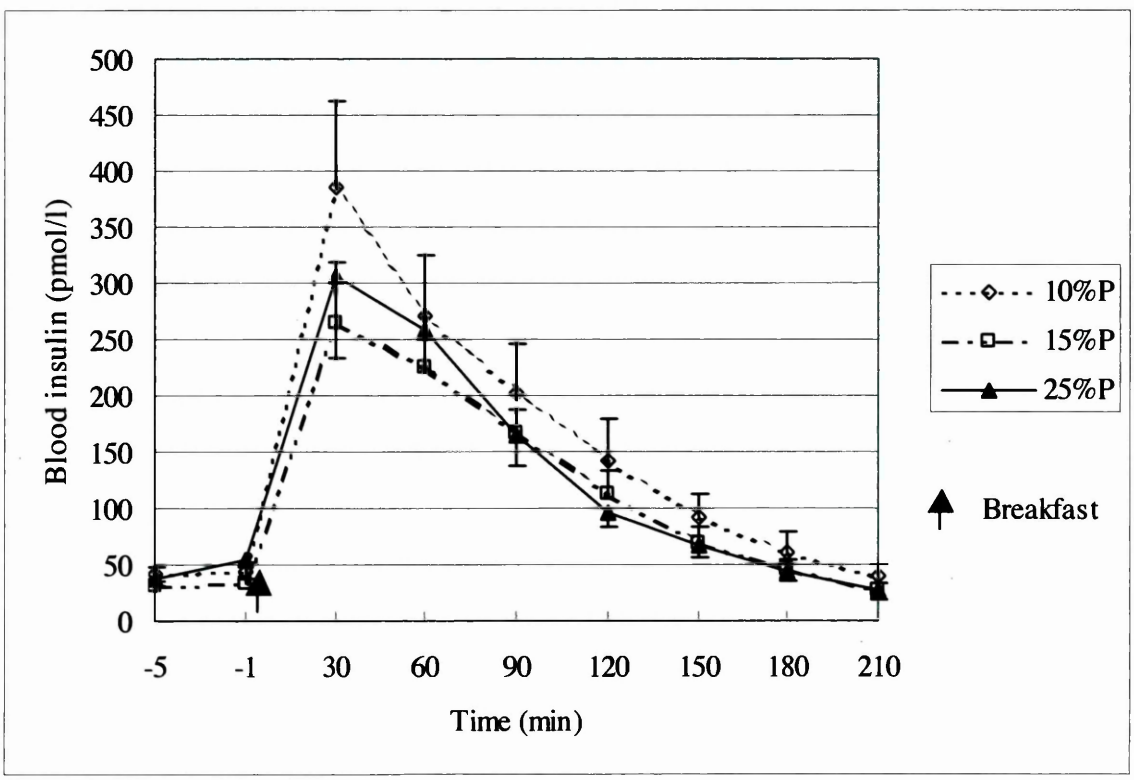


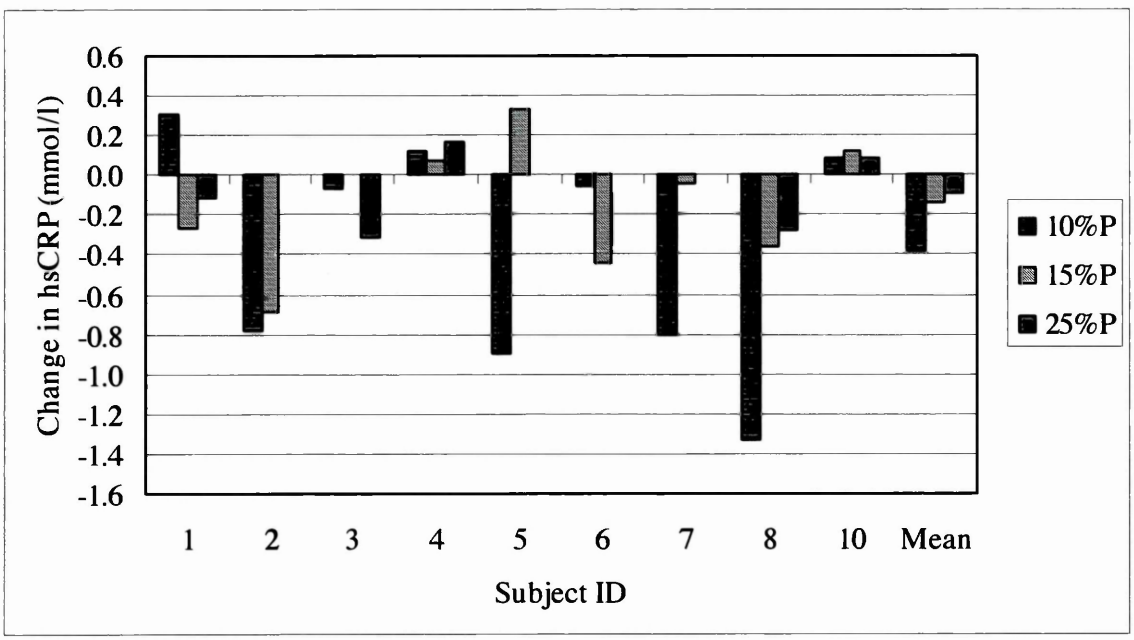

**Figure 4.3.42 Mean (SEM) blood insulin after a fixed-energy breakfast eaten at time 0**



4.3.3.8.6 HsCRP

Mean hsCRP at baseline was 1.13 mg/L (0.25 – 1.99) for the 10%P diet, 0.94 mg/L (0.15 – 1.91) for the 15%P diet, and 1.11 mg/L for the 25%P diet (0.29 – 2.61) which are all within the normal range. There was no difference in hsCRP concentration between diets on day 1 or day 4 of the study. hsCRP was lower on day 4 than day 1 during the 10%P diet (1.1 ± 0.2 mg/L versus 0.8 ± 0.2 mg/L, p = 0.048). This difference appears to be due to large individual differences in hsCRP as demonstrated in Figure 4.3.43. There was no difference in hsCRP between day 1 and day 4 of the study for the 15% P or the 25%P diets.

**Figure 4.3.43 Change in plasma hsCRP levels between day 1 and day 4 of each diet**




4.3.3.8.7 IGF-I and IGF-BP3

IGF-I concentration in subject 6 was considerably higher than the other subjects. All analyses were performed including and excluding subject 6. Results reported include all subjects unless removing subject 6 significantly altered the outcome.

There was no difference between the diets in subjects' fasting blood IGF-I concentration on day 1 or day 4. IGF-I was significantly higher on day 4 (mean 153.8 ng/ml ± 20 SEM) than day 1 (148.5 ng/ml ± 23) during the 25%P diet ($p = 0.03$). When subject 6 was removed from the analysis, the difference was increased, and the concentration of IGF-I was also significantly higher on day 4 than day 1 during the 15%P diet ($p = 0.02$). Individual differences in plasma IGF-I concentration are shown in Figure 4.3.44. The differences between day 1 and day 4 did not differ between diets ($p = 0.4$).

Post-prandial IGF-I concentrations were higher at 60 min ($p = 0.003$), 90 min ($p = 0.0007$), 120 min ($p = 0.02$) and 210 min ($p = 0.008$) during the 25% P diet as demonstrated in the post-prandial curves shown in Figure 4.3.45. AUC was significantly higher after the 25%P meal (27414 ng.min/ml ± 3959 SEM, $p < 0.0001$) and 15%P meal (26573 ng.min/ml ± 3970, $p = 0.01$) than the 10%P meal (24191 ng.min/ml ± 3959) when subject 6 was removed from the analysis, but there was no difference between diets with all subjects included ($p = 0.12$). The maximum concentration of IGF-I was higher after the 25%P meal ($p = 0.01$).

IGF-BP3 (IGF binding protein 3) was higher on day 4 than day 1 during the 10%P diet ($p = 0.001$) but the change in IGF-BP3 did not differ between diets ($p = 0.64$). Individual differences are shown in Figure 4.3.46. There was no difference in IGF-BP3 at any timepoint (Figure 4.3.47) after the test meal and the AUC did not differ between the diets ($p = 0.82$). There was no difference in IGF-BP3 for any other parameters analysed.

**Figure 4.3.44 Difference in plasma IGF-I levels between day 1 and day 4 of each diet**

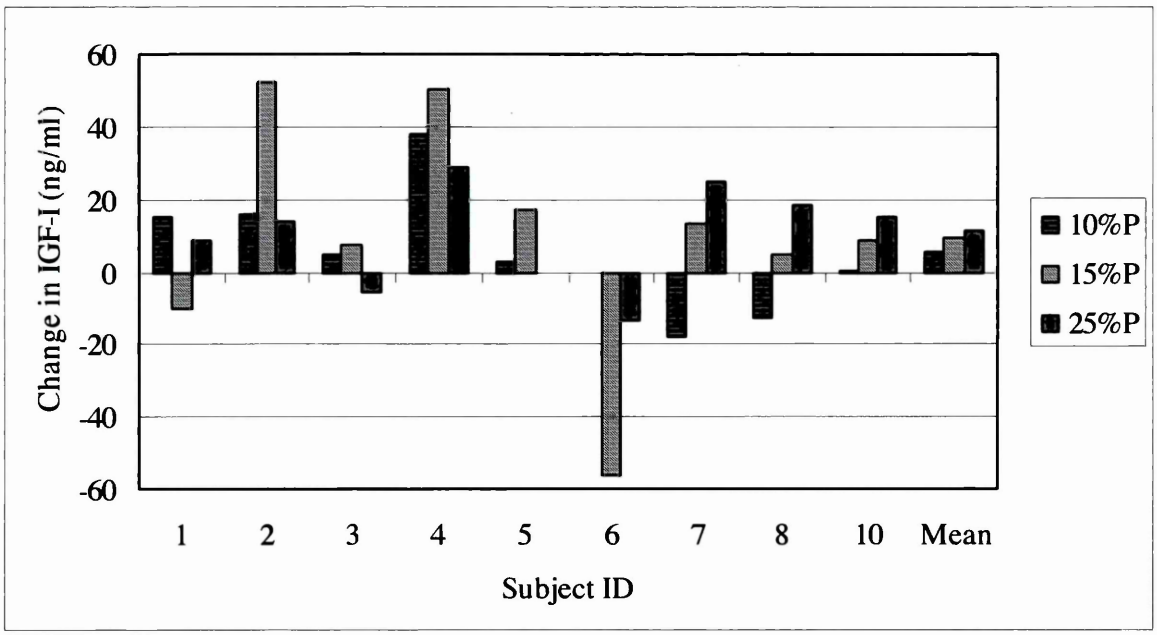
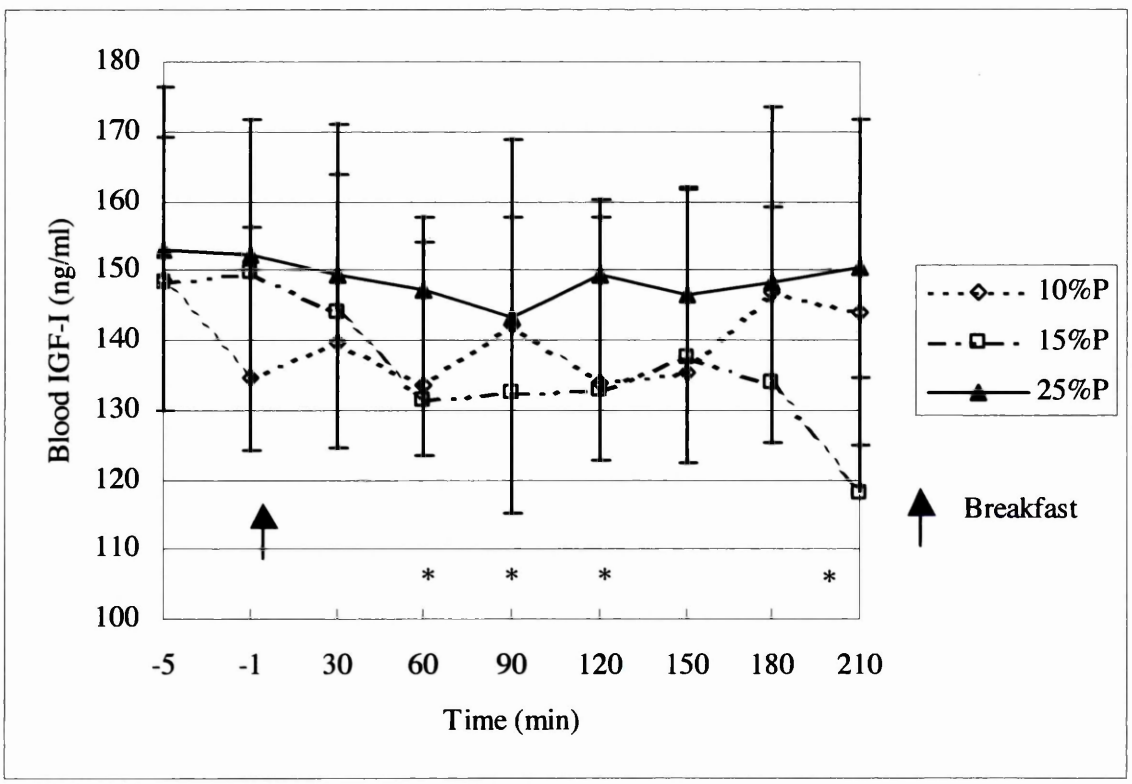



**Figure 4.3.45 Mean ± SEM blood IGF-I curves after a fixed energy breakfast eaten at time 0**




* indicates significant difference between 25%P and 15%P

**Figure 4.3.46 Difference in plasma IGF-BP3 levels between day 1 and day 4 of each diet**

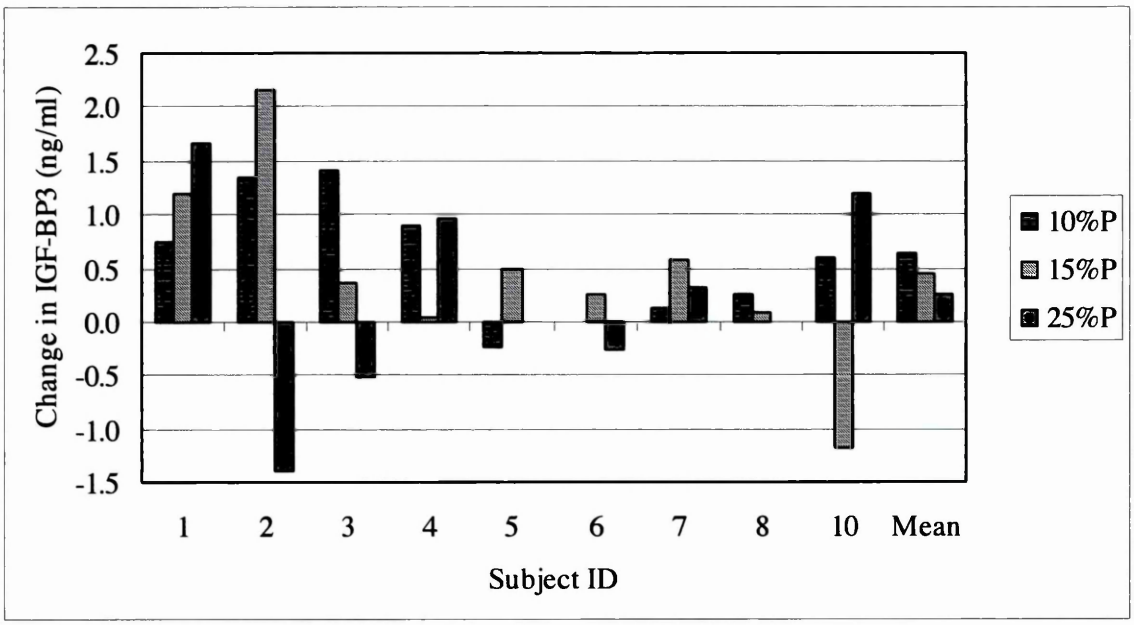
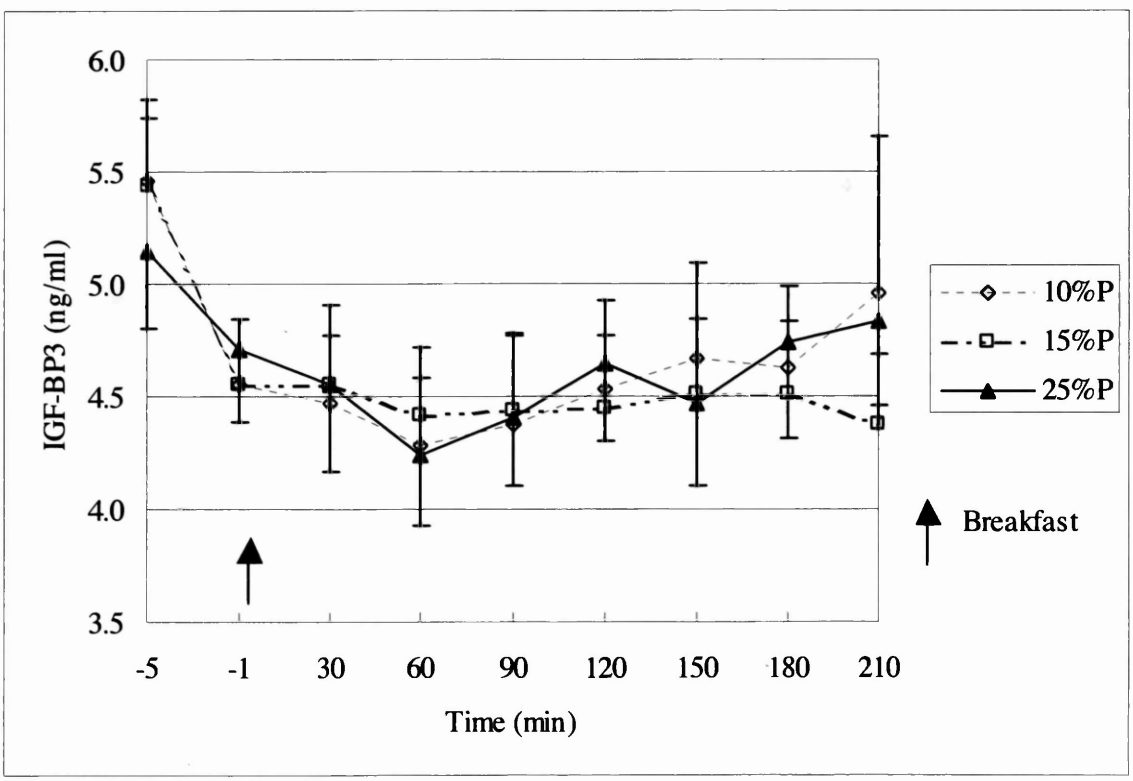



**Figure 4.3.47 Mean (SEM) blood IGF-BP3 curves over 3.5 hours after a fixed energy breakfast eaten at time zero.**



4.3.3.8.8 Blood lipids

There was no difference between diets in the total cholesterol concentration in the subjects' blood on day 1 or day 4 of the study visits. Total cholesterol was higher on day 4 than day 1 for each diet (10%P diet $p = 0.03$, 15%P diet $p = 0.005$, 25%P $p < 0.0001$), but there was no difference between the diets for the change in concentration between day 1 and day 4 (Figure 4.3.48).

LDL cholesterol concentration did not differ on day 1 or day 4 of each diet, or between day 1 and day 4. The change in concentration is shown in Figure 4.3.49.

HDL cholesterol concentration for all subjects is presented in Figure 4.3.50. The concentration on day 4 increased during the 25%P diet (0.25 mmol/l ± 0.01) and was significantly different to the change in concentration during the 10%P (-0.08 ± 0.06) ($p = 0.01$) and 15%P (-0.03 ± 0.06) ($p = 0.004$) diets.

Subjects' blood triacylglycerol concentration was higher on day 4 than day 1 for each diet (10%P - $p < 0.0001$, 15%P - $p = 0.001$, 25%P - $p < 0.0001$) but was significantly lower on day 4 with the 15%P (1.2 mmol/l ± 0.2, $P = 0.009$) and 25%P (1.2 mmol/l ± 0.2, $P = 0.011$) diets than the 10%P diet (1.5 mmol/l ± 0.2). The rise in triacylglycerol levels was lower with 15%P ($p = 0.012$) compared to the 10%P. Individual differences are shown in Figure 4.3.51.

**Figure 4.3.48 Change in blood total cholesterol levels between day 1 and day 4 of each diet**

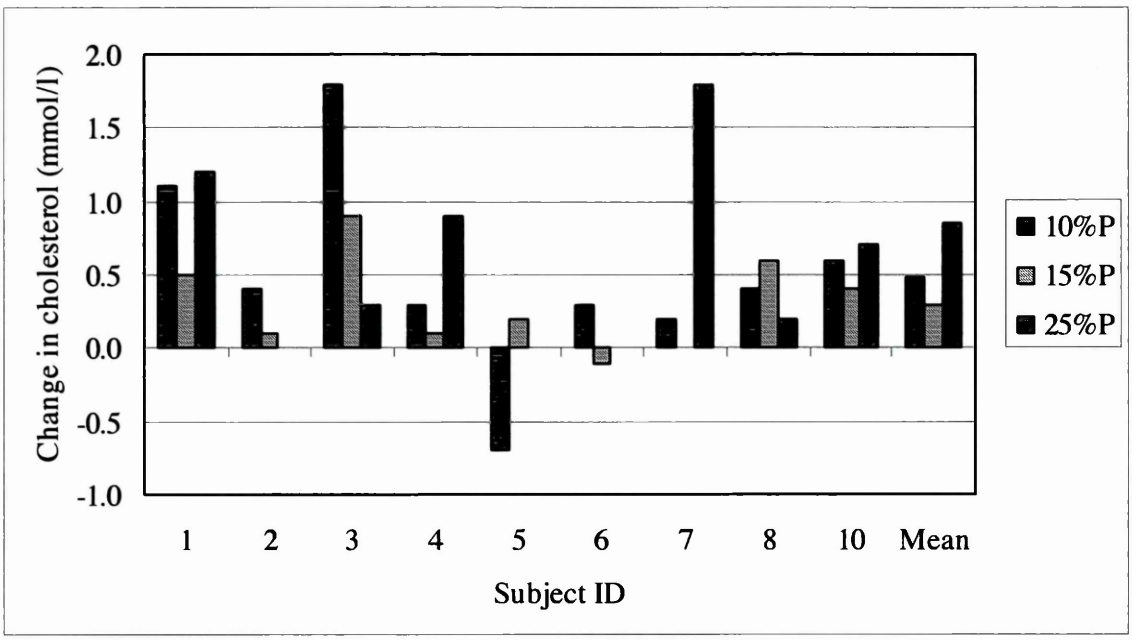



**Figure 4.3.49 Change in calculated LDL levels between day 1 and day 4 of each diet**

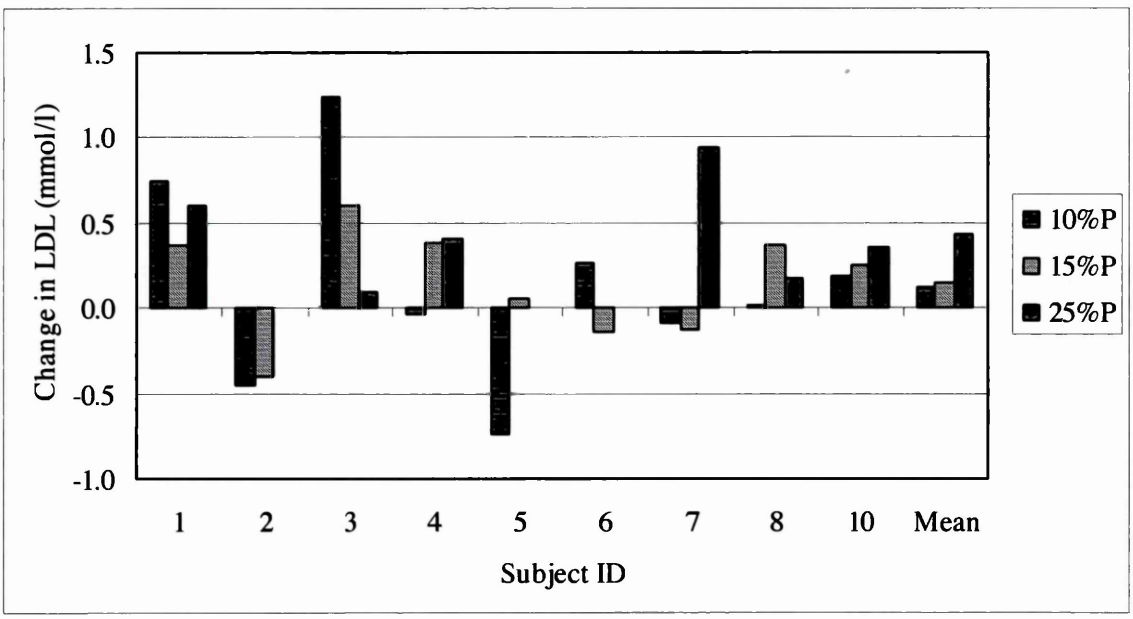


**Figure 4.3.50 Change in blood HDL levels between day 1 and day 4 of each diet**

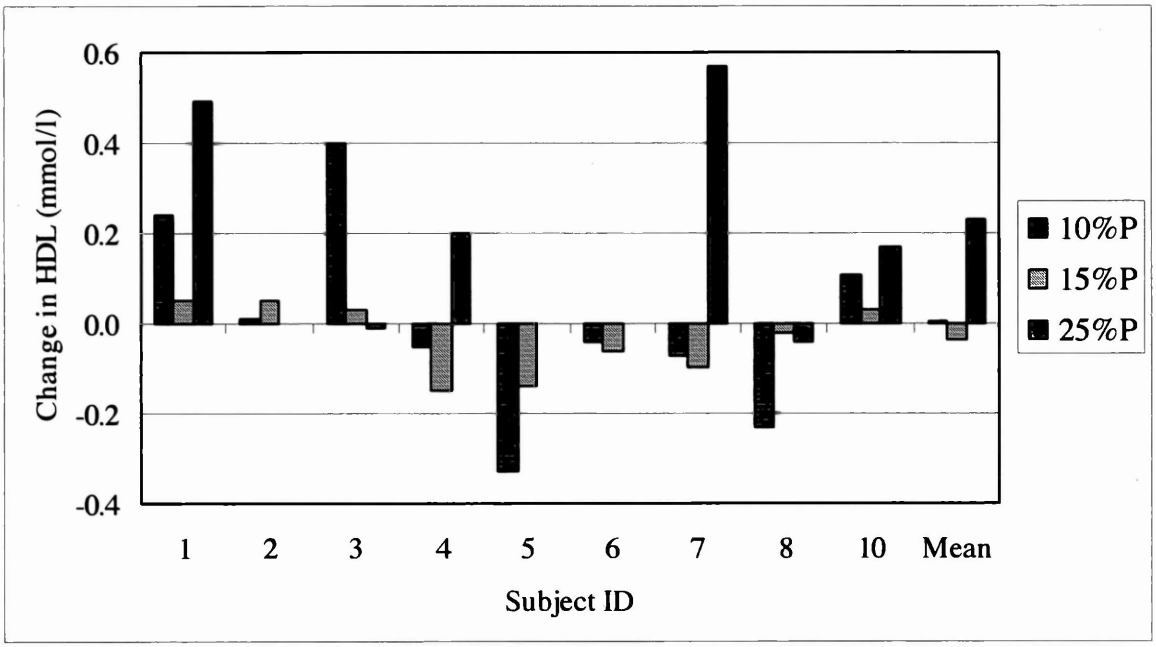
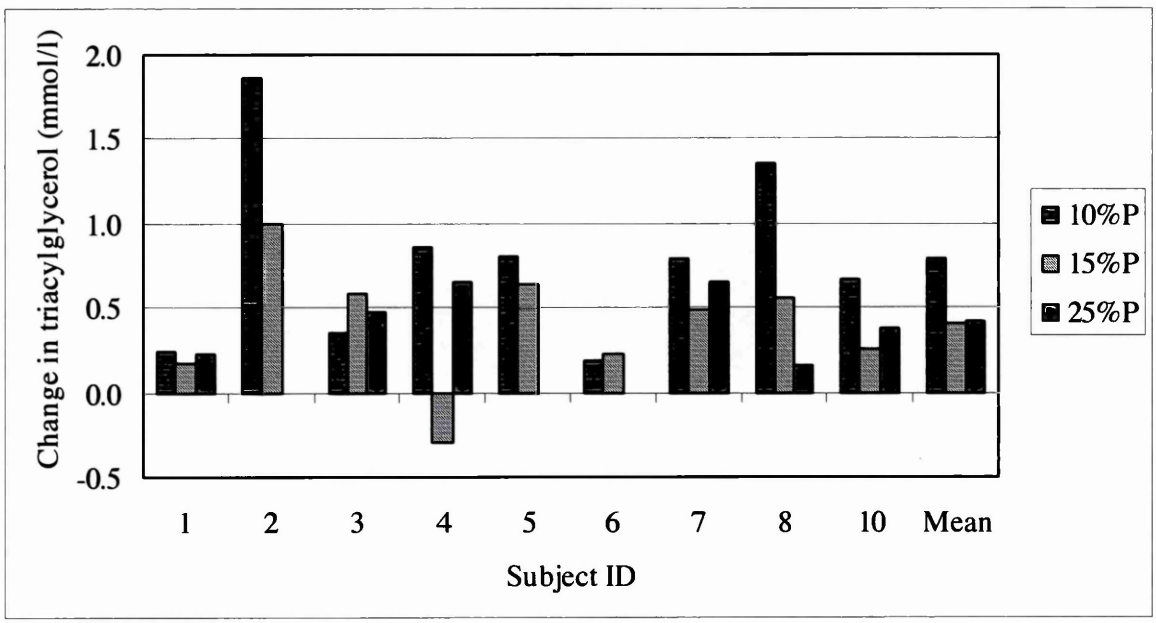



**Figure 4.3.51 Change in blood triacylglycerol levels between day 1 and day 4 of each diet**



## 4.4 Discussion

This study investigated the effect of diets of varying P:E ratios on *ad libitum* energy intake and appetite control mechanisms. The study was a crossover design, conducted under standardised conditions, to allow for within-subject comparisons and increased statistical power to detect any effects relative to a parallel design. The study diets were carefully planned and prepared with similar palatability to provide the intended differences in the proportion of total energy as protein.

This study reveals that a diet containing 25% energy as protein is associated with earlier meal satiation, increased fullness and a reduction in energy intake over 3 days. There was also a reduction in glucose excursions, an increase in post-prandial IGF-I and improvements in blood lipids. Taken together these data imply that a diet containing an increased P:E ratio may aid weight control and reduce metabolic risk.

There was however no clear effect of reductions in the P:E ratio. On a diet containing 10% energy as protein there was no effect on appetite sensations or energy intake, suggesting a non-linear relationship between P:E and the control of food intake or an inadequate decrease to detect a difference. However there was a clear deterioration in post-prandial glucose homeostasis with an increase in 24 h glycaemia with decreasing proportion of protein, indicative of an adverse effect on metabolic risk.

### 4.4.1 Effect of diets of varying P:E ratios on energy intake

This study demonstrated that when lean, healthy subjects were provided an *ad libitum* diet of varying P:E ratio over a three-day period, while confined to a metabolic suite, EI was reduced when protein was increased to 25% of energy (25%P) compared to a

'control' diet containing 15% energy as protein (15%P). The mean reduction in EI was 0.6 MJ/d, which, assuming no adaptation, equates to approximately 0.14 kg weight reduction per week or 3.7 kg over six months.

These values are broadly consistent with a previous study showing an immediate reduction in spontaneous consumption when participants were allowed to eat ad libitum on a diet containing 30% protein, following a 2 week period of controlled feeding to energy balance (Weigle *et al.*, 2005). Together these studies extend the evidence from short-term preload studies, discussed in Chapter 3, which consistently show that test meals high in protein reduce EI at a subsequent meal, compared to isoenergetic meals high in fat or carbohydrate. The present study, in which every meal was manipulated over 3 days, shows that the effect is robust and persists over time, without prompting autoregulatory compensation mechanisms. It may thus be of physiological significance in the longer-term control of body weight.

#### *4.4.1.1 The protein leverage hypothesis*

The protein leverage hypothesis predicts that when protein availability changes such that the ratio of protein:non-protein energy increases or decreases absolute protein intake is preserved (Simpson & Raubenheimer, 2005). The hypothesis predicts that when the available diet contains a higher percentage of protein, absolute protein needs are met earlier, without the need to consume as much non-protein energy, and total energy intake is reduced. Conversely when the available diet is low in protein more food must be consumed to meet the target protein requirements, increasing the total energy consumed. Modelling of this hypothesis indicates that small changes in the available protein have significant changes in the total amount of energy consumed.

The data from the present study support one component of the hypothesis, that an increase in the proportion of protein in the diet reduces total energy intake. It does not suggest that lowering available protein increases total energy intake, as no dose-response effect was demonstrated, in contrast to data from other animals, including mice (Sorensen *et al.*, 2008), rats (Simpson & Raubenheimer, 1997), carnivorous ground beetles and spiders (Mayntz *et al.*, 2005).

Humans may exert more conscious control over food intake than animals concealing their response to changes in the P:E ratio. It is also possible that the 10%P and 15%P diets were too similar in protein content to demonstrate a difference in EI (as may be suggested by the lack of a difference in nitrogen excretion from subjects after eating the 10%P and the 15%P diets). The only study to investigate the protein leverage effect in humans provided an *ad libitum* high or low-protein diet for two days after a two-day control period to 10 subjects (Simpson *et al.*, 2003). During the two investigation days, buffets of food high or low in protein were available at each meal. The five subjects in the high-protein group reduced their daily intake from 8.5 MJ/d to 5.6 MJ/d and consumed a diet that provided 45% of energy from protein. In the low-protein group subjects increased their intake from 7.5 MJ/d to 12 MJ/d and protein only provided 8.5% of total energy. The protein:non-protein energy ratios were of a much wider range than was used in the current study and the diets provided were unlikely to be sustainable for longer periods.

Diets comprised of 45% of protein or more have consistently been shown to reduce EI and increase satiety (Barkeling *et al.*, 1990; Poppitt *et al.*, 1998; Latner & Schwartz, 1999) (Porrini *et al.*, 1997) while meals containing less than 30% of energy from protein have provided less consistent results (Raben *et al.*, 2003; Vozzo *et al.*, 2003). As a diet comprising less than 30% of energy as protein is more likely to be sustainable and safe in

the long term, this area required further investigation and hence a 25%P diet was selected for the present investigations.

#### *4.4.1.2 Factors influencing energy intake*

In this study there was no effect on meal frequency. Instead the changes in energy intake occurred predominately within a meal, implying an effect on satiation. Prior research shows that satiation can be disrupted by the introduction of different foods (variety effect), the presence of others (social context) and competing tasks (distraction) (Hetherington, 2007). This study was highly controlled and took place in a metabolic suite to reduce the effects of these external influences on food consumption and to maximise the ability to detect physiological effects of satiety on energy intake at a meal and throughout the study visit. To limit the effects on food consumption of eating in the presence of others or with distractions, all meals were consumed in private booths with no other distracting events. Subjects were able to eat for as long as required, with dishes only removed once subjects indicated that they had completed their meal.

When a large variety of food is available, energy intake tends to increase (Hetherington, 2007). For this reason, there was no choice provided at each meal although several different dishes were presented throughout the day. Intake may also increase in response to large portions (Rolls *et al.*, 2006). However, the *ad libitum* nature of the study required a large amount of food to be offered at each meal to ensure that sufficient food was available for subjects to eat *ad libitum* and to discourage subjects from consuming the entire portion as it was presented, which may be habitual or perceived to be more socially acceptable. Similar portions were offered on each dietary treatment and subjects were broadly consistent in whether they over- or under-ate throughout the *ad libitum* eating

days in comparison to their calculated energy requirements, reinforcing the value of a within subject design. There was a slight, but not significant, tendency towards weight loss during the study visits suggesting that most subjects were not over-consuming because of the availability of large portions of food, and potentially that the lack of variety may have inhibited consumption. Subject 8 was a notable exception, as he consistently over-ate in comparison to his estimated energy expenditure consuming almost the entire portion at each meal. Removing these data from the analysis significantly affected the EI results and for this reason EI data were presented including and excluding the results from subject 8.

In contrast to the changes in EI observed when the P:E ratio of the diet was increased from 15%P to 25%P, EI did not differ when protein was decreased from 15%P to 10%P. Similarly urine nitrogen excretion was 93% higher during the 25%P diet compared to the 10%P diet and 57% higher than the 15%P diet but no difference was present between the 10%P and 15%P diets (despite a trend towards greater urine nitrogen excretion during the 15%P diet). This finding is somewhat surprising as, at lower intakes of protein, obligatory losses account for a greater proportion of nitrogen loss and urinary nitrogen excretion is anticipated to be lower than expected (Millward, 1997) rather than higher than predicted as demonstrated in this study. However Bingham *et al*, 1985, report that urinary nitrogen measurement underestimates dietary nitrogen at higher levels of protein. Whilst the mean urinary nitrogen (UN):dietary nitrogen (DN) ratio was 0.81 for 8 subjects consuming their normal diet over 28 days, the range was 0.7 to 0.9. The inter-individual and day-to-day variation accounted for a proportion of this range. However those subjects with the lowest protein intake were observed to have the highest UN:DN ratio (closer to 0.9) and subjects with greater protein intake produced a lower UN:DN

(Bingham & Cummings, 1985). In the present study a higher UN:DN could account for the higher than expected urinary nitrogen measured during the 10%P diet.

The P:E ratios selected for this study (10%P, 15%P, and 25%P) were designed to investigate the hypothesis that small changes in the proportion of protein available in the diet lead to a compensatory inverse change in fat and carbohydrate consumption and therefore in total energy intake. The similarities in EI and nitrogen excretion at the lower end of the normal range in this study, instead suggest a threshold effect where reducing protein intake has little effect on physiological mechanisms that regulate food intake. It is possible that greater reductions in protein availability may influence total energy intake. However, there would have been limited value in investigating the effects of a diet outside the range that could not be sustained from habitual foodstuffs.

The relatively short duration of this study may also have been a factor in control of EI. Dietary regulation is dependent on the duration of effect of feedback mechanisms to influence subsequent eating patterns. In contrast to laboratory rats where food intake is regulated by adjusting the interval between meals, in humans food intake is regulated by adjusting meal size and is determined by the length of time since the last meal and the amount of food remaining in the stomach (de Castro, 1988). The time period over which energy intake is regulated is less clear. Individual energy balance was measured in 12 men in an armed forces training establishment by directly measuring energy expenditure (measuring expired air in a Douglas bag) and weighing and recording all food consumed. There was no correlation between the mean expenditure on any one day and the intake on that day. However mean energy intake and expenditure was balanced over weekly and fortnightly periods. In particular, it was observed that there was a significant correlation between the mean daily expenditure and mean daily intake two days later. (Edholm *et al.*,

1955). In a further study, detailed analysis of seven-day food diaries showed that food consumed on one day has a small negative feedback on intake on the subsequent day and a much larger effect two days later. The effect continues on the third day but disappears by the fourth day (de Castro, 1998). These observations suggest that the three day time-period of *ad libitum* eating in this study was sufficient to maintain mean energy balance, however it is possible that additional days of dietary manipulation may have provided a more consistent response.

It has been argued that the regulation of macronutrient balance is more precise than for energy, with macronutrient intake on one day affecting intake up to two days later but not beyond (de Castro, 1998), and so the three days of the present study would have been adequate to establish a stable eating pattern. Given the consistency in daily food intake in participants in this study, where the patterns of response to the diets was established within a day and no differences in intake were identified between day 1 and 3 of each study week (Figure 4.3.6), the short duration of the study does not appear to have compromised the final outcome.

Inevitably, habitual eating habits and food preferences would have had some influence on total food consumption during the study. Our aim was to limit these factors as much as possible so that the physiological effects of the diets on energy intake could be assessed. However, to try to establish whether other factors influenced food consumption energy intake was analysed on each study day and for each meal and type of meal.

#### *4.4.1.3 EI per study day*

There was no consistent trend in EI over the three days for the three different diets, although there were differences in EI between individual days of each study visit. One

possible explanation relates to the energy density of the diets. The energy density of the food offered on day 2 was lower (5.4 kJ/g) than on day 1 (7.8 kJ/g) or day 3 (7.0 kJ/g). Increases in energy density are associated with an increased EI, especially in short-term laboratory studies (Stubbs *et al.*, 2000a) and may therefore account for the higher EI on days 1 and 3.

Palatability is another factor that may have affected EI on the different study days. Palatability questionnaires were only completed on day 3 of each visit (alongside the appetite questionnaires), in order not to overemphasise the importance of appetite and food intake. If subjects were aware that appetite and EI were being measured eating patterns may have been affected by conscious control mechanisms. In retrospect, ensuring similar palatability across the menus rather than just between individual dishes might have produced more convincing data. However, reducing variability may have been unacceptable to the subjects and affected energy intake later in the study week. Providing the diets in random order may have reduced the differences apparent on different study days. But, in view of the controlled nature of the study and the physiological investigations performed, it was important that the same meals were consumed at the same points throughout the study so that meaningful comparisons could be made.

EI increased on day 3 during the 15%P and 25%P compared to day 1 despite the same menu being offered. One possible reason for this increase in EI is that subjects may have been more comfortable with the metabolic suite environment by day 3 of the visit and so may have consumed more. The other possibility is that EI increased on day 3 to compensate for the reduced EI on day 2.

#### *4.4.1.4 EI per meal and per meal type*

To clarify more precisely when differences in EI were occurring, all meals and meal types were analysed separately. On the 25%P diet EI was reduced during meals (suggesting increased satiation), though its full impact was offset by an increase in snacks consumed. The reduction in EI at meals was apparent at both lunch and dinner and more snacks were consumed during both the daytime and the evening. Overall there was a reduction in EI at meals of 0.9 MJ/d compared to the 10%P and 15%P diets, a 10% decrease in EI which if sustained over a long period would lead to a substantial reduction in body mass. A 0.4 MJ/d increase in energy consumed as snacks lessened the effect of the 25%P diet to decrease EI at meals. It is difficult to extrapolate this finding to a free-living scenario, since snacks were readily available and there were few distractions during this study. Frequent snacking is likely to be atypical for some participants, although it must also be recognised that food access and availability are relatively high in many modern environments.

The discrepancy in the effect of the higher-protein diet on EI at meals and snacks may illustrate the difference between physiological signals of satiety and the influence of non-physiological factors that control eating behaviour. Meals were all consumed in private booths at discrete time periods whereas snacks were available throughout the day and were usually kept on the subject's desk where they could be consumed while other distracting tasks were being performed. An evening snack box was provided and snacks may have been consumed during other activities such as reading, working on the computer or watching television.

### 4.4.2 Effect of diets of varying P:E ratios on satiety

Satiety and satiation during a meal have an important role in energy balance. VAS questionnaires that record levels of satiety during and after a meal are a reliable indicator of the physiological response to eating (which may or may not translate to an effect on energy intake depending on environmental influences) (Flint *et al.*, 2000). Results of the appetite questionnaires completed during an *ad libitum* eating day support the hypothesis that diets higher in protein enhance satiety, and that an increase in satiety may be a mechanism for reducing energy intake during a meal and throughout the day.

VAS scores for fullness were consistently higher throughout the day during the 25%P diet, with the AUC 10% higher than during the 15%P diet. Fullness scores were significantly higher at 1100 h (two hours after breakfast), and 2000 h to 2200 h (two to four hours after dinner) suggesting that the effect of protein on satiety lasts for some hours after a meal.

The effect of increasing protein on satiety evident in this study is supported by a number of previous studies that have demonstrated an increase in satiety during or immediately after a meal (Stubbs *et al.*, 1996; Poppitt *et al.*, 1998) (Porrini *et al.*, 1995; Porrini *et al.*, 1997; Westerterp-Plantenga *et al.*, 1999; Oesch *et al.*, 2005; Harper *et al.*, 2007), throughout a day (Stubbs *et al.*, 1996) (Westerterp-Plantenga *et al.*, 1999), or over longer periods (Weigle *et al.*, 2005) (Westerterp-Plantenga *et al.*, 2004) (Lejeune *et al.*, 2005). Porrini *et al*, 1997 demonstrated that EI was reduced by 42% while subjects were consuming a high-protein meal compared to a high-fat meal, indicating the satiating properties of protein (Porrini *et al.*, 1997). EI was also reduced at an *ad libitum* meal immediately after a high protein preload meal but when a high-protein snack was consumed 2 h prior to a meal subjects reported increased fullness and reduced hunger

with no change in EI at the subsequent meal. Stubbs *et al*, 1996 (Stubbs *et al.*, 1996) and Harper *et al*, 2007 (Harper *et al.*, 2007) also reported higher satiety scores after a high-protein meal but no effect on EI at a test meal. The consistent reporting of higher satiety scores after high-protein meals (Porrini *et al.*, 1995; Poppitt *et al.*, 1998) (Latner & Schwartz, 1999) (Oesch *et al.*, 2005) supports the hypothesis that higher protein meals increase satiety and therefore may have a significant affect on EI when a high-protein meal is consumed.

The variation in the response to high-protein meals and subsequent eating patterns suggest that the physiological control of satiety may be overridden by external influences that affect the amount of energy consumed over an entire day. This hypothesis is supported by the results from this study that indicate that high-protein meals promote satiety during the meal. Although this diet reduced total EI for the entire day, the effect was offset by the non-physiological factors that influence snacking or grazing (Gatenby, 1997).

### 4.4.3 Dietary manipulation

The study diets were designed to be as consistent as possible in appearance, taste, energy density and fibre while maintaining the appropriate macronutrient composition. Food was selected that would be familiar to the UK population. Habitual diets of the subjects (comprising 15% of energy from protein, 36.9% from fat and 48% from carbohydrate) were broadly comparable in terms of macronutrients, to the standard UK diet (16.5% of energy from protein, 35.5% from fat and 48.1% from carbohydrate) (NDNS, 2003) and the 'control' diet used in the study was the same as habitual protein intake (15% of energy). Fat (30% of energy) was lower, consistent with dietary recommendations for

good health, and consequently the proportion of energy provided as carbohydrate (55%) was higher than a standard UK diet.

Standard supermarket ingredients were used in the preparation of all food and unusual tastes such as spices were limited. The three meals (with lunch and dinner having two courses) and three to four snack options per day provided a variety of dishes on each study day to allow for different food preferences. However at each meal only one dish was available to prevent the effect of a larger variety of food stimulating energy intake (Rolls *et al.*, 1981; Stubbs *et al.*, 2001). All meals were eaten in a private booth to reduce any influence from other study subjects or metabolic suite staff.

Table 4.2.3 and Table 4.2.4 summarise the composition of each meal for the different P:E ratios. Energy density was within 1 kJ/g for all the main meal dishes. Where food was baked, such as the muffins and scones, or had few ingredients, such as the sandwiches, maintaining energy density proved to be more difficult. In these dishes the macronutrient composition was prioritised and was able to be kept within 1% of the intended composition in all dishes except the peanut butter sandwiches. The proportion of energy from main meals was significantly higher than from snacks (Figure 4.3.13) so is unlikely to have significantly altered total energy intake. Fibre was adjusted in all recipes by adding bran or altering ingredients and was constant for all main meals. Both fibre and energy density are important determinants of energy intake (Rolls *et al.*, 2005) and satiety (Hulshof *et al.*, 1993), therefore to attempt to isolate the effect of the macronutrients on these factors it was necessary to create meals that were as similar as possible except in their macronutrient content.

The consistency of meals is demonstrated by the results from the palatability questionnaire. While there was no evidence of a difference in palatability between diets there were some individual differences between meals. This variation was difficult to avoid as standard grocery items were used to prepare the meals and not all differences in the ingredients could be removed. There did not appear to be a relationship between palatability scores and EI. The 25%P breakfast meal was rated the most filling and the 15%P meal was rated the most enjoyable, however EI was slightly higher during the 10%P meal (ns). At lunch, the 25%P meal was rated the tastiest and the most satisfying and EI was the lowest perhaps reflecting the satiating properties of the higher-protein meal, as discussed in section 4.4.2. The dinner meal produced the largest differences. The 15%P meal was tastier, more pleasant and more enjoyable than the 10%P meal and consistent with this EI was highest. The 25%P meal was rated more enjoyable than the 10%P meal but EI was the lowest.

The 10%P meals were the least familiar to the subjects and palatability scores tended to be lower for the main meals. It may be that the slightly lower palatability at these meals reduced EI and constrained any underlying drive to increase EI as P:E ratio decreased. Conversely the 25%P diet had higher palatability ratings at the main meals but EI was consistently lower. Higher palatability and familiarity with the study meals may have exaggerated EI during the 15%P meals. In subsequent collaborative studies we have undertaken more formal pretesting of diets to reduce the effect of differences in palatability (Gosby *et al.*, 2010).

### 4.4.4 Effect of diets of varying P:E ratios on metabolic risk factors

#### *4.4.4.1 Body mass*

There was no difference in body mass between the treatments which is to be expected with such small differences in EI over just three-days. Since subjects were confined to a calorimeter (see Chapter 5) on day 4 (the morning after their third *ad libitum* eating day) the final weight was not taken until the morning of day 5. A fixed-energy diet was provided on day 4 designed for weight maintenance. In practise some subjects ate less than the calculated energy requirements so the delay in making the final weight may have compromised the quality of this measure. Additionally there was the potential for changes in hydration to confound body mass measurements. Subjects were not accustomed to the drier atmosphere of the constantly air-conditioned metabolic suite environment and although fluid intake was *ad libitum* throughout the study, fluid replacement varied between subjects. Some subjects reported headaches that may have been attributable to inadequate fluid replacement and there was some evidence of inadequate fluid balance on clinical examination.

There was a trend towards a reduction in body mass on each of the study diets which is likely to be atypical but does indicate that subjects did not significantly overeat during the study despite the availability of a large amount of *ad libitum* food. There may be a number of explanations for this trend. It is possible that the 60 min daily walk was more activity than subjects were accustomed to, although all subjects reported performing some physical activity, with a mean of more than 60 min of moderate and light physical activity. Since the remainder of the day was entirely sedentary it seems unlikely that usual physical activity was exceeded. Instead it is perhaps more likely that subjects found

the food less pleasant than their habitual diet or that intake was constrained by low variety of food offered.

#### *4.4.4.2 Body composition*

Changes in body composition over the short time-period of each study week were small and below the sensitivity of measurement techniques (Jebb *et al.*, 1993). This may explain the discrepancies observed between the different methods. Changes in fat mass and fat-free mass measured by Bod Pod and DXA differed which probably relates to the different assumptions of these two methods.

Bod Pod measurements assume a constant hydration of lean mass but it is likely that hydration status and glycogen stores in this study changed over the course of the study week and possibly changed differentially with each study diet. DXA assumes that tissue consists of bone, fat and lean soft tissue. Lean soft tissue is calculated as the difference between body mass and fat and bone components. Changes in hydration have been demonstrated by modelling overhydration states. Water and saline were added to meat of known fat and lean composition and fat estimation errors were calculated after the resulting models were scanned using DXA (Pietrobelli *et al.*, 1998). The results clearly show that DXA fat estimation errors occur with added fluid, however the direction of error depended on the type of fluid added. Additional normal saline predicted fat mass to be lower than actual fat mass while water added to the model predicted fat mass to be higher than actual fat mass. The error increased with a higher proportion of added fluid, however it is unlikely that in healthy subjects hydration states would change substantially to significantly affect measurements. When hydration changes by 1-5% as might be

expected in healthy people the estimated error of measurement of fat mass is <1% (Pietrobelli *et al.*, 1998).

The Bod Pod measured a significant decrease in fat mass during the 10%P and 15%P diets that did not occur during the 25%P diet. Contrary to these data, DXA measurements showed a reduction in fat mass only during the 25%P diet. Fat-free mass appeared to rise slightly during the 15%P diet as measured by Bod Pod but did not differ during any of the diets when measured by DXA.

When these discrepancies were examined further there was a clear difference in the relationship between measurements in fat mass and fat-free mass with changes in body mass which may reflect the manner in which the Bod Pod and DXA are able to measure changes in water flux and hydration status and changes in glycogen stores. A trend for change in fat mass versus change in body mass is evident when fat mass is measured by Bod Pod (Figure 4.4.1) but not when measured by DXA (Figure 4.4.2) reflecting the greater accuracy of the Bod Pod. The opposite occurs when fat-free mass is measured. There is only a weak relationship between fat-free mass and body mass measured by Bod Pod (Figure 4.4.3) but DXA shows a strong relationship between change in lean mass versus change in body mass (Figure 4.4.4). This increase seems implausible, since increase in lean tissue apparently exceeded the increase in body mass.

**Figure 4.4.1 Change in fat mass versus change in body mass measured by Bod Pod**

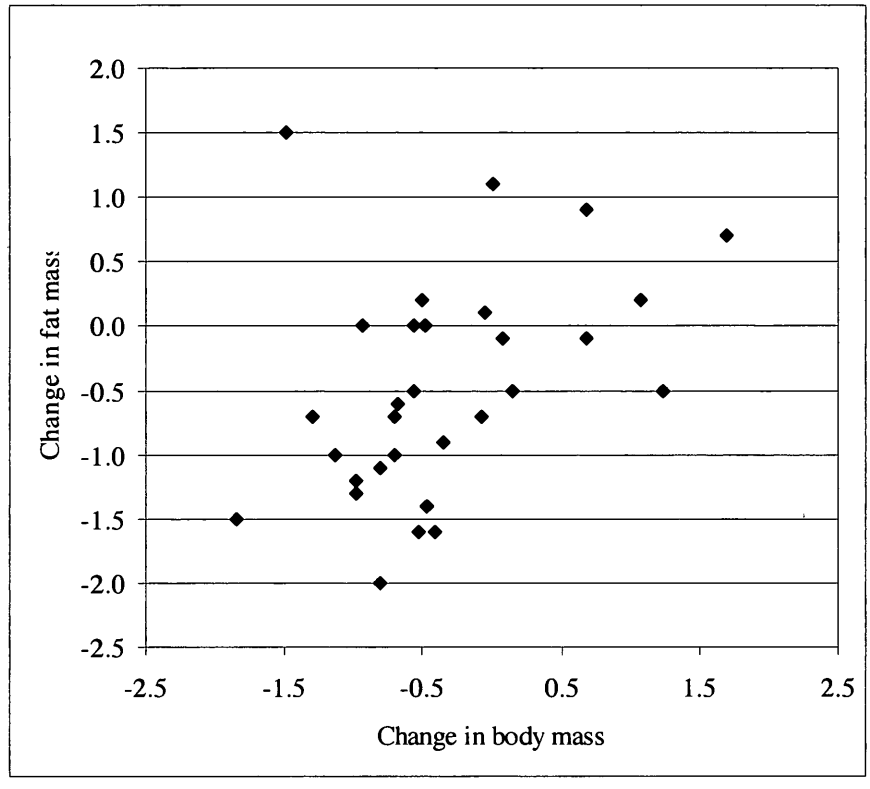



**Figure 4.4.2 Change in fat mass versus change in body mass measured by DXA**

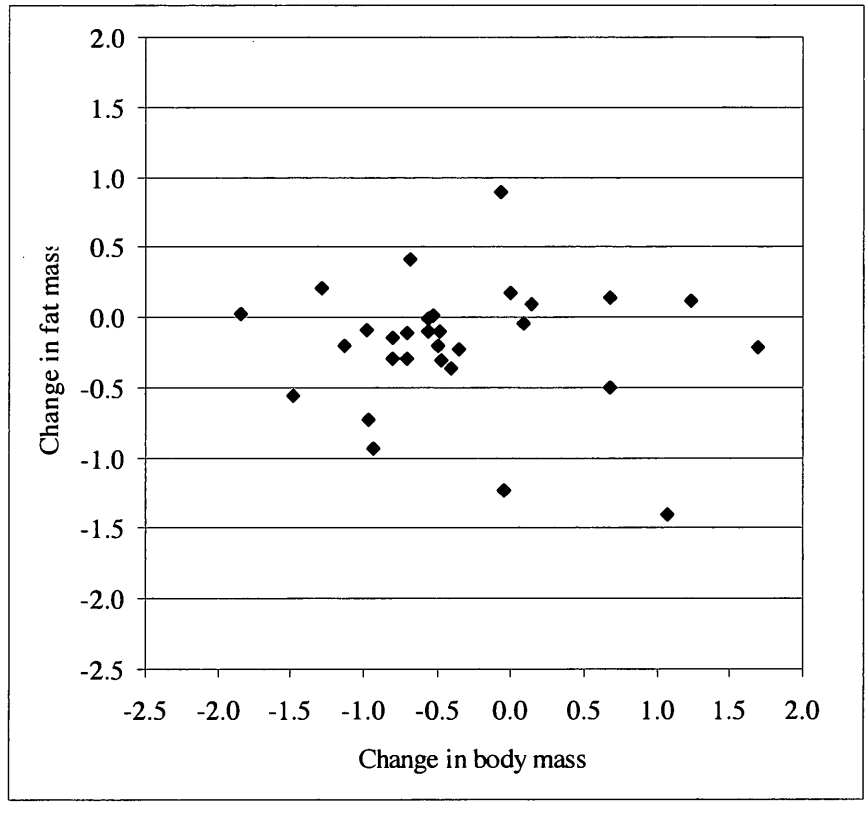


**Figure 4.4.3 Change in fat-free mass versus change in body mass measured by Bod Pod**

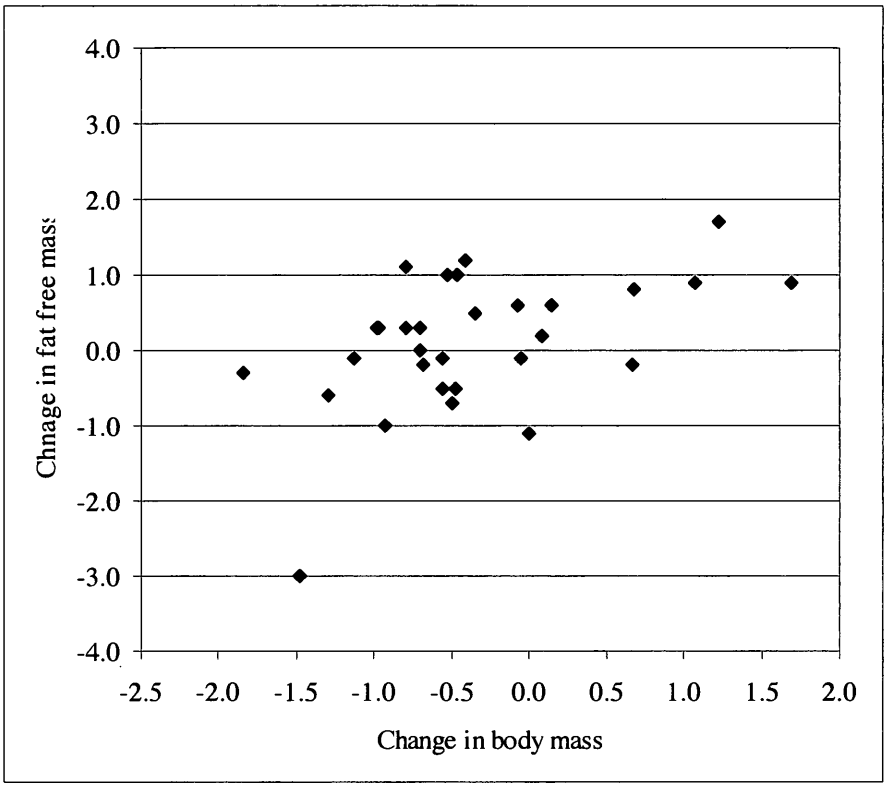
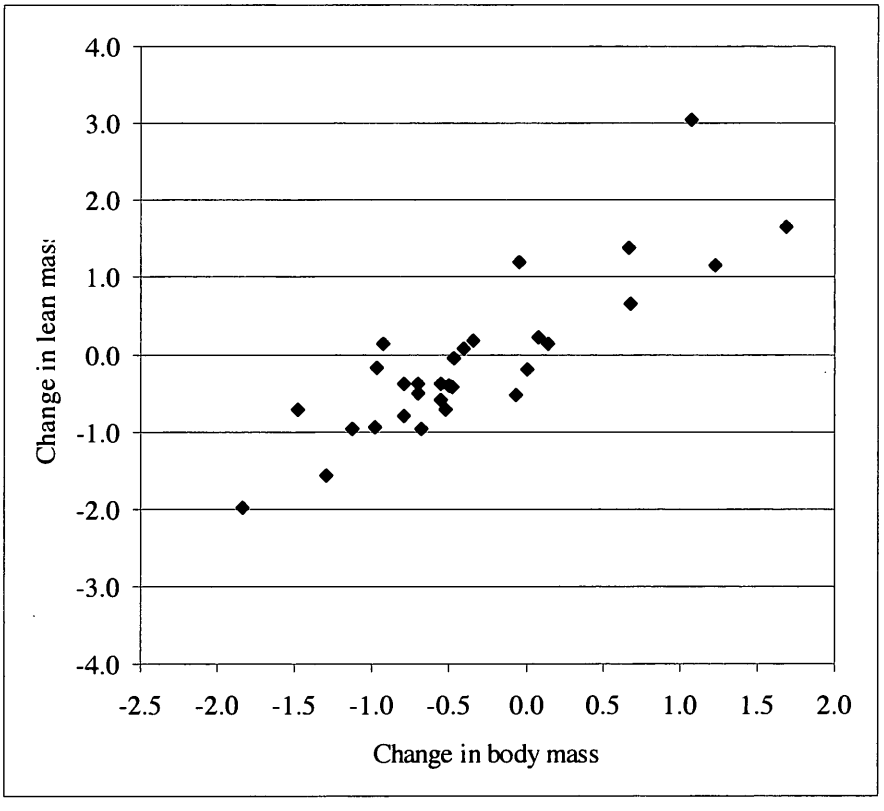



**Figure 4.4.4 Change in lean mass versus change in body mass measured by DXA**



The lack of a consistent and plausible measured effect of the diets on body composition in this study is due to the short duration of each diet and time between measurements. Over a longer period a higher percentage of protein in the diet has been reported to preserve lean mass and reduce fat mass in the context of weight loss (Layman *et al.*, 2003) (Farnsworth *et al.*, 2003) (Brinkworth *et al.*, 2004) or during weight maintenance after weight loss (Westerterp-Plantenga *et al.*, 2004). It is possible that in the short term, a diet higher in protein and lower in carbohydrate appears to lower lean mass because of lower hydration of lean tissue. Lower carbohydrate intake lowers glycogen stores in muscle and liver. As glycogen is lost, fluid is also lost from these tissues (Kawamori, 1999) increasing errors in the accurate measurement of fat and fat-free mass.

#### *4.4.4.3 Blood pressure*

There was no effect of the diets on BP during the study weeks. Blood pressures of individuals were variable throughout the study, possibly due to different stressors perceived by the subjects. Given the short duration of each diet it is not surprising that no significant change in blood pressure was identified but a longer-term effect of the protein content of the diet on blood pressure remains possible.

#### *4.4.4.4 Lipids*

Evidence is accumulating to suggest that diets with higher-protein:non-protein energy may improve the blood lipid profile in spite of initial concerns that diets higher in protein, particularly animal protein which is often associated with lipids rich in saturated fatty acids, may be detrimental. Higher total lipids and lipids with more saturated fatty acids are associated with earlier onset of atherosclerosis and an increased risk of heart disease, and may therefore counteract any benefit obtained from weight loss from a high-protein diet. However, prospective data from 80082 women in the Nurses Health Study supports

the benefits of a diet higher in protein on ischaemic heart disease. The relative risk of heart disease was 0.74 in women who reported high intakes of protein compared to those who ate less protein (Hu *et al.*, 1999).

During a 10-week weight loss study 24 women randomised to a 30% or 16% protein diet (with fat constant at 30%) reduced their serum cholesterol by 10% in both diets consistent with the 8% weight loss in both groups (Layman *et al.*, 2003). The high-protein group also had significant reductions in triacylglycerols and the ratio of triacylglycerol:HDL. These reductions in lipids were obtained despite the high-protein diet containing more than twice the dietary cholesterol of the low-protein diet. A reduction in blood triacylglycerol concentration has been reported in similar long-term studies of high-protein and low-carbohydrate diets (Foster *et al.*, 2003) (Samaha *et al.*, 2003) but not over such a short period as in the present study.

Over the four-day period of each diet in this study, there was an unexpected rise in total blood cholesterol that was not diet dependent. A rise in serum cholesterol during periods of stress has been reported (Sane, 1978) and it is possible that the cholesterol levels in this study may have risen after having spending one night in a calorimeter and being confined to a metabolic suite for the previous three days. Despite this HDL cholesterol increased only during the 25%P diet, and the change in triacylglycerol concentration was significantly lower during the 15%P and 25%P diets. Elevated serum triacylglycerol concentration indicates the presence of triacylglycerol-rich lipoproteins which can penetrate the arterial intima, become trapped in the arterial wall and promote development of atherosclerosis. Recently two prospective cohort studies have identified a strong association between triacylglycerol levels and cardiovascular events (in 26509 women in the Women's Health Study followed for 11 y) (Bansal *et al.*, 2007) risk of MI,

IHD and death (in 7587 women and 6394 men followed for 26 y) (Nordestgaard *et al.*, 2007). Non-fasting triacylglycerol levels may be particularly important in the development of atherosclerosis and a stronger association was found when triacylglycerol levels were measured in the non-fasted state.

#### *4.4.4.5 Glucose*

Hyperglycaemia is a central component of the metabolic syndrome, and elevations in post-prandial glucose concentrations are associated with a significantly increased risk of developing type 2 diabetes and cardiovascular disease. In this study two methods were used to investigate the effects of the dietary intervention on post-prandial and 24 h glucose patterns. First, interstitial glucose was measured using continuous glucose monitoring to investigate whether diets of different P:E ratio produced differing effects on 24 h glucose patterns when diets were consumed *ad libitum* in a similar manner to a free-living individual, and in the more controlled fixed-energy environment. Second, fasting blood glucose concentrations were measured at the start and end of each study week to identify whether the diets produced longer lasting effects on overnight glucose metabolism. Post-prandial blood glucose curves were also measured to investigate the effect of the meals and to compare results to that obtained with the continuous glucose monitor, as although this method of measuring glucose levels is commonly used in managing people with type 1 or type 2 diabetes, it has been used infrequently in healthy subjects with normal glucose levels.

Glucose levels recorded by the CGMS demonstrated a clear and sustained beneficial effect of a higher-protein diet on 24 h glucose levels and post-prandial glycaemic excursions, both important factors in reducing the risk of metabolic disease. During both the *ad libitum* and fixed energy intake days glucose levels were consistently lower in the

pre and post-prandial periods with the 25%P diet than lower protein diets. The significant dose-response effect of the protein content of the diets on post-prandial maximum glucose concentration during the fixed-energy day is evident in Figure 4.3.39. The difference between the glucose curves is clearer than during the *ad libitum* eating day as all meals were eaten at the same time, no snacks were provided during the day, and each subject ate the same amount of energy on all three visits. In contrast, during the *ad libitum* eating day eating patterns were more variable and attenuated the subtle effect of diet composition. There was little difference between the glucose concentrations during the 10%P and 15%P for any period analysed. The unexpectedly lower EI during the 10%P than the 15%P diet may account for this lack of difference. Despite a lower EI during the 10%P diet, glucose concentrations were the same as during the 15%P diet. If EI had been similar during the 10%P and 15%P diets, or lower during the 15%P diet as predicted by the protein leverage hypothesis, glucose levels would be expected to have been higher during the 10%P diet in a similar pattern to the fixed-energy eating day.

There was no evidence of a difference in fasting blood glucose levels measured at the start and end of each study week, which is perhaps not surprising over such a short time-period period and in lean, non-diabetic subjects. Glucose concentration in the fasted state is maintained by the more complex process of hepatic gluconeogenesis, which is regulated by changes in glucose, insulin, free fatty acids, and glucagon (Kawamori, 1999). Although hepatic glucose production is very responsive to changes in insulin concentrations, changing the whole system would expect to take longer than three days.
The predominant mechanism for improving post-prandial glycaemia after a meal lower in carbohydrate and higher in protein is a reduction in the initial glucose load. Whether the effect on glucose levels is primarily due to the change in protein or the reciprocal changes in carbohydrate is uncertain and hence the effect of high-protein diets in a free-living state

depends on the wider dietary context. It has been proposed that glucose homeostasis improves during a high-protein diet independent of carbohydrate intake and is mediated through up-regulation of liver gluconeogenesis during the fasted state and increased glycogen synthesis after a meal (Westerterp-Plantenga *et al.*, 2006).

Surprisingly, little difference between the diets was demonstrated in post-prandial blood samples taken after the fixed-energy breakfast. This finding differs somewhat to the CGMS glucose concentration recorded at the same time and is most plausibly explained by the methods used to measure glucose. Firstly, the CGMS measures interstitial glucose at 5 min intervals. Blood glucose samples were taken at 30 min intervals to allow for the time required to collect and process a sample and to limit the total blood taken to an ethically acceptable quantity. It is therefore possible that the blood samples may not have been taken at the maximal peak or trough level and underestimate the post-prandial curve. Secondly, changes in interstitial glucose concentration lag behind those of blood glucose concentration by 4-10 min (Boyne *et al.*, 2003) depending on insulin-mediated glucose uptake to peripheral tissues and peripheral sensitivity to insulin which differs at different sites and between subjects (Aussedat *et al.*, 2000). The magnitude of an increase in interstitial glucose after a glucose load is reported to be 70% of that in plasma glucose suggesting that CGMS measurements may underestimate peak glucose values. During the subsequent decrease in glucose, interstitial glucose decreases faster than plasma glucose due to insulin stimulated peripheral glucose uptake. Differences between interstitial and plasma glucose are therefore greatest when glucose is rapidly increasing (Kulcu *et al.*, 2003). It is possible that in this study the CGMS results indicate a much larger difference in post-prandial glucose concentrations that may have been identified in the blood if plasma samples were taken at more frequent time intervals. It has been argued that interstitial glucose levels may be more clinically important as they more closely reflect

cellular glucose concentrations, where most complications secondary to hyperglycaemia arise (Boyne *et al.*, 2003).

The improvement in post-prandial and 24 h glucose concentration with a diet higher in protein demonstrated in this study is supported by a number of previous studies. Increasing protein to 30% of energy in 8 subjects with type 2 diabetes for five weeks in a crossover study resulted in a reduction in mean 24 h glucose and glycohaemoglobin (9.8% in the control 15%P diet compared to 7.6% in the high-protein diet) (Gannon & Nuttall, 2004). A further study in 10 obese subjects with type 2 diabetes demonstrated a normalisation of mean 24 h glucose levels and a reduction in HbA1c from 7.3% to 6.8% (Boden *et al.*, 2005). In both of these cases, the experimental diets were high in protein but very low in carbohydrate. Blood glucose levels would therefore be expected to be lower after a period of a low-carbohydrate diet and the effect of the increased protein cannot be isolated from the change in carbohydrate. A more substantial effect would also be expected in subjects with type 2 diabetes, as blood glucose levels are higher and less tightly controlled at baseline.

An effect on blood glucose after a single meal has also been demonstrated. 23 overweight to moderately obese women were provided with an isoenergetic high-carbohydrate or high-protein snack comprising 22% P, 59% C, 19% F and 750 kJ (Williams *et al.*, 2006). Mean maximum blood glucose concentrations were 5.7 mmol/l after the high-protein snack and 6.8 mmol/l after the high-carbohydrate snack. Similarly this study was performed in overweight subjects in whom a larger post-prandial blood glucose excursion may be expected. It is notable that in this study of lean subjects with no increased risk for diabetes or metabolic disease the post-prandial maximum blood

glucose differed by 2.5 mmol/l after dinner (6.8 mmol/l during the 25%P diet and 9.3 mmol/l during the 10%P diet) and the mean 24 h glucose differed by 0.7 mmol/l.

The difference in 24 h glucose profiles during the *ad libitum* eating day is notable given that extensive research comparing the action of foods differing in glycaemic index on post-prandial glycaemia has produced mixed results. A previous crossover study investigating glucose profiles with diets differing in their glycaemic index in 19 hyperinsulinaemic women was not able to identify any differences during *ad libitum* eating after 12 weeks of the intervention diet (Aston *et al.*, 2010). In a shorter study 10 healthy adults were given instructions to consume one low-glycaemic index food with each meal for seven days. CGMS glucose profiles were lower after the seven days of consuming low-glycaemic index foods than at baseline (Brynes *et al.*, 2005). The difference in results was predominantly due to a lower overnight and fasting glucose in the latter study, with little difference observed between the two diets post-prandially. In contrast, in the present study, small changes in the P:E ratio produced significant differences in 24 h glucose profiles, suggesting that increases in the proportion of dietary protein may be a more effective strategy than altering glycaemic index to improve glycaemic control.

#### *4.4.4.6 Insulin*

The subjects' post-prandial blood insulin concentrations were independent of the diets consumed, which is not surprising given that the samples were taken at the same timepoints as the blood glucose concentration which did not differ between diets. Insulin is secreted from the pancreas in response to dietary intake and blood glucose concentration. Protein eaten with glucose stimulates insulin secretion significantly more than similar quantities of protein or glucose eaten separately, resulting in a reduction in

blood glucose (Nuttall *et al.*, 1984) and protein may stimulate insulin secretion to assist in the control of blood glucose. Over a longer duration insulin sensitivity has been reported to increase, particularly insulin-mediated glucose uptake in skeletal muscle when lean body mass is preserved during weight loss (Westerterp-Plantenga *et al.*, 2006). If it had been possible to measure insulin concentration throughout the day in this study, it is likely that a reduction in post-prandial and mean insulin concentrations in a similar pattern to the changes in glucose would have been observed.

#### *4.4.4.7 Insulin-like Growth Factor-1 and IGF Binding Proteins*

IGF-I is important in the regulation of growth. More than 90% of IGF-I in the blood is bound to IGF binding proteins, predominantly IGF-BP3. Epidemiological evidence suggests that IGF-I may increase the risk of prostate, colorectal and premenopausal breast cancers, all cancers linked to indices of growth such as body mass index, physical activity, and rapid growth in early life (Renehan *et al.*, 2004), while a low IGF-I concentration is associated with the development of osteoporosis, heart disease and glucose intolerance (Larsson *et al.*, 2005) (Sandhu *et al.*, 2002).

IGF-I appears to have an intrinsic role in glucose metabolism. IGF-I is structurally similar to insulin, and the IGF-I receptor, through which the biological action of IGF-I is mediated, is a tetramer with two α and two β subunits similar to the insulin receptor (Ogawa, 1999). IGF-I production is primarily regulated by growth hormone but insulin enhances IGF-I production. Infusions of IGF-I reduce blood glucose and insulin concentrations (Dunger & Acerini, 1997) and, in animals, inactivation of the IGF-I gene, resulting in a marked decrease in circulating IGF-I, is associated with hyperinsulinaemia and insulin insensitivity. A longitudinal study of 615 normoglycaemic subjects measured IGF-I at baseline and after 4.5 y follow-up. The odds ratio for developing impaired

glucose tolerance or type 2 diabetes with an IGF-I concentration >152 μg/L was 0.50 compared to a concentration <152 μg/L (Sandhu *et al.*, 2002).

IGF-I levels are predominantly determined by genetic factors and sex but physical activity and diet may also modify circulating IGF-I. Serum IGF-I levels are increased with higher-protein intake, particularly dairy protein (Norat *et al.*, 2007) (Larsson *et al.*, 2005). IGF-I levels measured in 2109 women were positively related to protein intake but IGF-BP3 did not appear to be related to dietary factors (Norat *et al.*, 2007). In another study of 226 men who undertook 14 dietary telephone questionnaires over one year reporting 24 h food intake to estimate long-term diet, increased consumption of protein was associated with higher IGF-I concentrations.

These epidemiological data support the results reported in this study. IGF-I increased during the 25%P study visit but not during the 10%P visit, and post-prandial IGF-I was higher after the higher-protein meal than the lower-protein meal, suggesting that protein affects IGF-I concentrations immediately after a meal and over a four-day period. In agreement with Norat *et al* (2007) there was no evidence of a change in IGF-BP3 with a change in dietary protein in the present study (Norat *et al.*, 2007).

#### *4.4.4.8 Gastrointestinal hormones*

There is increasing recognition of the importance of gastrointestinal hormones in the control of satiety, energy intake and glucose metabolism. Altering the post-prandial secretion of gastrointestinal hormones may be one mechanism by which dietary protein exerts an effect on these factors. In this study, the analysis of these hormones may have been sub optimal, since new assays became available over the course of the study which required different processing schedules. When the blood sampling protocol was initially

established, there was no ability to measure the active components of ghrelin, GLP-1 or PYY. The blood samples for hormone analyses were all stabilised immediately with aprotinin while subsequent assay development recommended the addition of a DPP-IV inhibitor to stabilise the active components of the hormones. This procedure was performed where necessary when the samples were defrosted to limit further degradation of the active hormones. There was a good post-prandial response for all the hormones measured, supporting the validity of this method.

Ghrelin levels rise before a meal and fall shortly after a meal, as observed in this study. Ghrelin increases appetite and food intake by increasing the number of meals initiated rather than increasing meal size. The response of ghrelin to changes in macronutrient intake is not clear, as previous investigations have led to contradictory results. In this study, ghrelin concentrations fell to a similar level after each of the study diets. The differences in concentrations at baseline were not significantly different but there appeared to be a larger fall after the 25%P meal, than after the 10%P meal, which followed a fairly flat profile. The higher concentrations of ghrelin at baseline would account for the trend to a larger AUC after the 25%P meal. Reports that carbohydrate intake is the most effective macronutrient at suppressing post-prandial ghrelin concentration (Karhunen *et al.*, 2008) may explain the lowest ghrelin concentrations in the 10%P diet containing the highest carbohydrate content. However prolonged suppression of ghrelin after a high-protein breakfast compared to a breakfast high in carbohydrate has been observed (Bowen *et al.*, 2006b; Bowen *et al.*, 2007) as has no effect of protein intake on plasma ghrelin responses (Smeets *et al.*, 2008) (Lephart *et al.*, 2004). One reason for the contradictory data on ghrelin measured in subjects eating meals differing in macronutrient composition may be that the most potent suppressor of ghrelin release is ingested energy rather than a specific macronutrient component of the meal.

Such mechanisms would account for the similar suppression observed after all of the (isoenergetic) meals in the present study.

Plasma GLP-1 concentrations rise rapidly after a meal and, while carbohydrates are a strong stimuli of GLP-1 release (consistent with the incretin role of GLP-1) protein appears to produce an even greater release of GLP-1 (Karhunen *et al.*, 2008). When 19 subjects consumed diets rich in protein (32% of energy), carbohydrate (12% of energy from protein) or fat (11% of energy from protein) the greatest post-prandial response and 5 h AUC occurred after the high-protein meal (Raben *et al.*, 2003). Similarly, in 28 obese men who were investigated for four hours after a test beverage, whey protein (85% of energy from protein) produced a higher and more sustained increase at 120 min in GLP-1 than fructose or glucose (11% of energy from protein) (Bowen *et al.*, 2007). In the present study there was a trend to a dose-response relationship in the maximal GLP-1 concentration and the AUC. A sustained increase in GLP-1 at 120 min after the 25%P meal suggests a greater effect of protein on GLP-1 release that may have been more apparent if a the meal had been higher in total energy or was comprised of a greater proportion of protein (as was used in the previous two studies). This dose-response relationship is logical given the incretin effect of GLP-1 and the reduction in plasma glucose that was evident in the 24 h glucose curves during the 25%P diet.

PYY is released in response to food intake in the distal part of the GI tract and, while secretion is usually observed within 30 minutes of ingestion of a meal, the long period of elevated PYY release present in this study is consistent with previous investigations of post-prandial PYY release, due to a ongoing release of PYY from the gut. PYY concentration was lowest in the 25%P diet at baseline and remained lower throughout the post-prandial period, with a trend towards lower AUC compared to the 15%P and 10%P

diets. Batterham *et al* (2006) demonstrated a greater PYY response to a high protein meal than high fat or high carbohydrate, but the high protein meal contained 65% of energy from protein and a larger response to the meal would therefore be expected (Batterham *et al.*, 2006). In contrast, Smeets *et al* (2008) in a similar design to the present study, demonstrated no difference in PYY concentrations of 30 subjects who consumed a high-protein (25% of energy from protein) and a low-protein (10% of energy from protein) lunch (Smeets *et al.*, 2008). Similarly the data in this study suggested a lower PYY response to the 25%P meal than the 10%P meal.

Food intake activates the parasympathetic nervous system and stimulates secretion of pancreatic polypeptide (PP) from the pancreas. Levels are low while fasting but increase throughout the phases of digestion (Karhunen *et al.*, 2008) as was seen in the present study. The mean AUC and the maximum concentration was higher during the 25%P and 15%P diets than the 10%P diet. Stimulation of PP by protein-rich meals has been observed in 19 obese children provided with a low-protein (0.2 g/kg) or high-protein (2.0 g/kg) meal. Blood samples collected for three hours after the meal demonstrated a larger peak response to the high-protein meal (581 ± 127 pg/ml) than the low-protein meal (302 ± 93 pg/ml) (Zipf *et al.*, 1983). Interestingly high-protein meals have been shown to stimulate PP secretion to a greater extent than low-protein, high-carbohydrate meals even during sham feeding (when subjects tasted the meals and then spat out the food) (Witteman *et al.*, 1994). PP appears to have an important role in satiety and energy balance in the short and long term (Koska *et al.*, 2004). 90-minute infusions of PP in 14 lean subjects produced a reduction in energy intake (2.4 MJ) compared with saline (2.7 MJ), and hunger scores were lower during the PP infusion (Jesudason *et al.*, 2007). Therefore the greater stimulation of PP secretion with the 25%P and 15%P diet compared

to the 10%P diet observed in the present study is likely to be one of the mechanisms that resulted in increased satiety and reduced energy intake in the higher-protein meals.

### 4.4.5 Summary

This study has shown that increasing the P:E ratio of the diet from 15% to 25% over a three-day period of *ad libitum* intake reduces total energy intake, particularly energy consumed during meal times and later in the day, suggesting an impact on satiation. The reduction in energy intake was supported by an increase in reported satiety during the 25%P diet, particularly fullness scores and prospective consumption of food. Neither a change in energy intake or an effect on satiety was evident when the P:E ratio of the diet was reduced to 10%. Although predictions from the protein leverage hypothesis suggest that a change in P intake from 15%P to 10%P is sufficient to result in a subsequent increase in energy intake, it is possible that in humans the difference between the diets is too small to alter EI significantly because habitual eating habits and non-physiological factors also influence eating habits and energy intake

The reduction in EI on diets with higher P:E ratio may arise from the enhanced satiety and, although not seen in this study because of its short duration, promote weight loss.

This mechanism is supported by data showing enhanced secretion of gastrointestinal hormones. Higher PP secretion in response to the high-protein diet is consistent with an increase in satiety, and the trend to increased GLP-1 with increasing protein would promote glucose disposal and reduce post-prandial glycaemia.

There was a clear effect of modifying the P:E ratio on 24 h glucose profiles and post-prandial glycaemic excursions. The lower, flatter glucose curves of the 25%P diet in comparison to the 15%P and 10%P diets are consistent with a reduction in metabolic risk if the diet was followed in the long term. IGF-I concentration increased over the duration of the study and when measured post-prandially, consistent with the change in glucose metabolism during the higher-protein diet. The intrinsic link between insulin and IGF-I also suggests that insulin action is likely to have been altered over the duration of the study, although such changes were not detected during the post-prandial measurements performed after breakfast. These changes, together with a reduction in triacylglycerols suggest that a modest increase in the P:E ratio may attenuate the development of atherosclerosis and cardiovascular disease.

By demonstrating a reduction in energy intake and enhanced satiety when consuming a diet of 25%P compared to a standard diet of 15%P, this study supports the increasing evidence that a higher P:E ratio reduces energy intake and improves weight control. The favourable effects of the higher-protein diet on markers of metabolic risk in addition to improved weight regulation may provide additional beneficial effect by reducing the risk of developing metabolic diseases and assist in the management of people with established glucose intolerance or type 2 diabetes.

# 5 Chapter 5 Measuring the effect of manipulating the protein:non-protein energy ratio of the diet on energy expenditure and substrate oxidation.

## 5.1 Introduction

Manipulating the protein:non-protein energy ratio of the diet over short and medium term time frames has been shown to promote weight loss and weight maintenance. Evidence presented in Chapter 4 suggests that increasing the P:E ratio may increase satiety and reduce hunger, and subsequently reduce energy intake. The degree to which energy intake is reduced over the long term, and the contribution of the enhanced satiating properties of protein to sustained weight maintenance are uncertain. It also remains possible that dietary protein influences energy balance by altering energy expenditure.

A high-protein meal increases diet-induced thermogenesis and may therefore lead to higher daily energy expenditure (see 1.5.4). Atkins (1998) argued that a diet high in protein confers a metabolic advantage: an increase in body mass reduction per kilojoule compared to diets of similar energy content with different macronutrient composition (Atkins, 1998). This metabolic advantage is hypothesised to be predominantly due to an increase in energy expenditure, which promotes weight loss (Feinman & Fine, 2003). In randomised controlled trials, weight loss was disproportionately greater in subjects following a high-protein, low-carbohydrate diet over six months compared to a high carbohydrate, low fat diet despite similar energy intake (Samaha *et al.*, 2003) (Foster *et al.*, 2003). If energy intake was indeed similar, another factor must contribute to the

differences in weight loss between the diets. According to the energy balance equation, energy balance, and therefore weight maintenance, is the sum of energy intake and energy expenditure, so the weight loss observed in these studies must be due to increased energy expenditure, through increases in BMR, thermogenesis or voluntary physical activity.

Short-term studies of high-protein diets have suggested an increase in energy expenditure compared to low-protein diets. Energy expenditure was measured in a calorimeter at baseline and on day four of each intervention period in 12 overweight and obese men provided with four days of a diet rich in pork protein (29% of energy from protein), soy protein (28% of energy from protein) or carbohydrate (11% of energy from protein). 24 h EE was 3% higher with the pork protein diet than the high-carbohydrate diet and 2% higher with the soy protein than the high-carbohydrate diet. BMR, SMR and DIT were all higher after the pork diet than the carbohydrate diet. One limitation of this study was that the diet was consumed at home for three days before the subjects entered the calorimeter, reducing the ability of the investigators to monitor all food consumption. Additionally the diets were not equally matched for energy intake, energy density or fibre content, which may have influenced the final day of investigations (Mikkelsen *et al.*, 2000).

A further study measured EE in a group of twelve lean women over 24 h in a calorimeter. The study diets were composed of 30% of energy from protein, 40% carbohydrate and 30% fat or 10%, 60% and 30% respectively, and meals were provided at home for three days prior to the investigation days. This adaptation period reduces complications arising from sensory effects on DIT. While there was no difference in total EE during the 24 h period, DIT and SMR were greater during the high-protein diet than the low-protein diet (Lejeune *et al.*, 2006).

DIT is the component of energy expenditure that is most commonly associated with altering dietary protein. Eight lean and overweight women consumed isoenergetic diets high in protein (29% protein, 61% carbohydrate, 10% fat) or fat (9% protein, 30% carbohydrate, 61% fat) over 36 h whilst energy expenditure was measured in a room calorimeter. DIT was higher during the high-protein diet (1295 kJ/d) than the high-fat diet (931 kJ/d) ($p > 0.05$) (Westerterp *et al.*, 1999) and was correlated with measures of satiety, perhaps suggesting that post-prandial increases in oxygen consumption and body temperature may regulate satiety (Westerterp-Plantenga *et al.*, 1999). DIT may be affected by sensory stimulation and palatability of the meal as well as the familiarity of the meal (Westerterp-Plantenga *et al.*, 1999) and it is possible that these factors contributed to the rise in DIT in this study in addition to a macronutrient effect. However, despite the increase in DIT, there was no difference in TEE or SMR, which questions whether there is any substantial effect of high-protein diets that may lead to increased EE in the long term.

The thermic effect of a food is the increase in energy expenditure above baseline following consumption of food and drink. It is dependent on the digestion, absorption and disposal of ingested nutrients, and therefore is influenced by the composition of food consumed. The body has limited storage capacity for protein and it must therefore be metabolically processed immediately following a meal. Whole-body protein synthesis increases after a meal and the high cost of peptide bond synthesis during this process leads to an increase in thermogenesis after a protein-rich meal which is greater than isoenergetic consumption of fat or carbohydrate. Robinson *et al* (1990) determined rates of energy expenditure and protein turnover in seven men who received hourly isoenergetic high-protein or high-carbohydrate meals over 9 hours. The thermic responses to the high-protein meals (9.6% of energy intake) were greater than to the high-carbohydrate meals (5.7%). Whole-body nitrogen turnover was higher during the high-protein meals and

calculations based on ATP requirements were used to estimate the rate of protein synthesis. Robinson concluded that 68 ± 3% of the thermic response to a high-protein meal could be accounted for by increases in protein synthesis compared with the fasting state (Robinson *et al.*, 1990).

Gluconeogenesis and ureogenesis have been proposed as additional mechanisms for the higher thermic effect of a protein-rich meal (Feinman & Fine, 2003). The production of glucose from non-carbohydrate precursors is energetically costly. A high-protein diet may stimulate gluconeogenesis, particularly when the carbohydrate content of the meal is very low, in order to ensure adequate glucose is present for brain, CNS and red blood cell function. In response to the utilization of protein stores to produce glucose, protein synthesis must occur to maintain nitrogen balance. The combined effect of greater gluconeogenesis to meet the obligate demand for glucose and the subsequent increase in protein turnover may therefore contribute to higher post-prandial energy expenditure after a high-protein diet.

Veldhorst *et al* (2009) investigated the effect of a high-protein, carbohydrate-free diet on gluconeogenesis in ten lean men. In a crossover design, subjects received an isoenergetic diet comprising 30% of energy from protein, 0% from carbohydrate and 70% from fat or 12%, 55% and 33% of energy from protein, carbohydrate and fat for 1.5 days (Veldhorst *et al.*, 2009). EE was measured in a room calorimeter and gluconeogenesis was calculated using an infusion of isotopically labelled glucose to determine endogenous glucose production and fractional gluconeogenesis. Fractional gluconeogenesis and therefore absolute gluconeogenesis was higher during the high-protein diet (171g/24 h) than the high-carbohydrate diet (145g/24 h) and the contribution of increased gluconeogenesis to increased EE was calculated at 42%. There were no difference in subjects' blood insulin

levels after the two diets to account for the increase in gluconeogenesis as has been previously observed (Veldhorst *et al.*, 2009). Of the components of total EE, RMR was significantly higher in the high-protein diet at 8.46 MJ compared to 8.12 MJ during the high-carbohydrate diet, but there was no difference in TEE, SMR or DIT between the two diets.

In addition to total energy expenditure measured in a calorimeter, it is possible to use oxygen consumption and $CO_2$ production, together with a measure of nitrogen loss, to calculate substrate oxidation - the contribution of individual fuels (protein, fat and carbohydrate) to energy expenditure and, if intakes are known, the partitioning of fuels over the period investigated (see 2.4.1 for details). The balance between food intake and the oxidation of the components of a meal may therefore predict whether energy balance is maintained or whether there is any imbalance. The oxidative hierarchy for macronutrients suggests that, because of the body's requirements for glucose and the limited storage capacity of carbohydrate, the need to maintain carbohydrate balance is prioritised. Carbohydrate oxidation is therefore closely matched to intake. Protein follows in the hierarchy. Protein oxidation is well matched to intake and, in the short term, body protein mass is unchanged. Fat oxidation shows little relation to intake, as, in healthy metabolism, there is a large ability to store fat in adipose tissue. During positive energy balance fat oxidation is inversely proportional to the oxidation of other substrates, due to the prioritisation of carbohydrate and protein. Reduced fat oxidation may therefore result in an accumulation of body fat and long-term body mass gain (Jebb *et al.*, 1996).

In two studies previously discussed, isoenergetic diets were provided in order to maintain body mass and substrate oxidation was measured while subjects were in the calorimeter (Westerterp *et al.*, 1999), (Lejeune *et al.*, 2006). In the latter study during the high protein

diet subjects were in a positive protein balance and a negative fat balance, which may indicate fat loss in the long term. The low protein diet was conversely associated with a positive fat balance (Lejeune *et al.*, 2006).

Westerterp *et al.* (1999) reported significant differences in substrate utilization compared to intake of each substrate when subjects were fed in energy balance (Westerterp *et al.*, 1999). Energy expended on a high-protein diet (29% P, 61% C, 10% F) was derived 18% from protein, 70% from carbohydrate and 12% from fat, whereas during the high-fat diet (9% P, 30% C, 61% F) 11% of energy expended was derived from protein, 51% from carbohydrate and 38% from fat. There was therefore a positive protein balance during the high-protein diet and a large positive fat balance on the low-protein diet. A negative carbohydrate balance during the high-fat diet may have resulted from a high-carbohydrate meal provided the day prior to the investigation days. The lack of a close relationship between macronutrient intake and oxidation, observed in this study, demonstrates the difficulty in extrapolating data from the short-term experimental environment to the long-term. The data indicates that time for adaptation to a diet is required before substrate utilization is adjusted to ingestion, suggesting that the effect of the diet on macronutrient oxidation persists to a certain extent.

Evidence therefore exists that a high-protein meal is associated with a greater thermic effect relative to isoenergetic fat or carbohydrate, due primarily to the high energy cost of increased protein turnover and gluconeogenesis. Whether this increase in post-prandial energy expenditure increases 24 h EE, or substrate oxidation to maintain body mass, is less clear. It has been argued that an increase in EE explains the observed beneficial effects of a high-protein diet on weight loss and maintenance. Investigations in a

controlled setting of the longer-term effect of increasing the protein:energy ratio of the diet on EE and substrate oxidation are required in order to test this hypothesis.

### 5.1.1 Objective

The objective of this study was to measure the effect on energy expenditure of short-term changes in the P:E ratio (where protein provided 10%, 15% or 25% of energy) under isoenergetic conditions.

## 5.2 Methods

This study was performed at the Wellcome Trust Clinical Research Facility (CRF) between March 2007 and December 2007. The study was approved by Cambridge Local Research Ethics Committee in November 2006 (Ref No. 06/Q0108/181), by the Addenbrooke's NHS Foundation Trust Research and Development Committee in January 2007 (Ref: A090877), and the Scientific Advisory Board for the Wellcome Trust Clinical Research Facility at Addenbrooke's Hospital in November 2006.

### 5.2.1 Subjects

The subjects who participated in this study also participated in the study described in Chapter 4. Details of the methods of recruitment of subjects and the eligibility criteria are discussed in 4.2.1.1.

#### *5.2.1.1 Sample size*

Based on previous research in the calorimeters at the CRF a sample size of 10 was calculated to be sufficient to detect a change in 24-hour energy expenditure of 2%, when participants were studied on non-consecutive nights (Napolitano *et al.*, 2011).

### 5.2.2 Study design

Energy expenditure was measured in a room calorimeter with subjects consuming a fixed EI diet in which protein was 10%, 15% or 25% of energy from protein, 30% from fat and 60%, 55% or 45% from carbohydrate. Each measurement period was preceded by a three-day period of *ad libitum* intake on a diet of matched P:E ratio.

All subjects had participated in the study discussed in Chapter 4, where for three days *ad libitum* food had been consumed, manipulated to provide 10, 15 or 25% of energy from protein. After the evening meal on day three, subjects returned to the CRF from HNR. A peripheral cannula was inserted into an antecubital vein according to the method discussed in Chapter 2 and flushed. The cannula was subsequently flushed prior to the subjects going to sleep, on waking, and after every blood sample.

Subjects entered the room calorimeter at 2000 h and the door was closed until the morning of day five (after 37 h). EE was measured throughout this entire time in the calorimeter.

*Ad libitum* eating conditions continued until 2230 h, when subjects were required to hand back their snack box to CRF staff. No food was subsequently available until the morning of day 4 when the fixed-intake day commenced. Subjects completed VAS questionnaires

until 2200 h. Interstitial blood glucose measures were taken according to the protocol described in 4.2.3.5.

BMR measurements were recorded between 0800 h and 0900 h each day. Subjects were woken at 0700 h to pass urine, and returned to bed to rest. During the BMR measurement subjects were required to lie supine and still throughout the measurement time.

Fasting blood samples were taken at 0930 prior to the breakfast meal, and at half hour intervals after the breakfast meal until 1330. Samples were taken through the cannula that had been inserted the previous night. Subjects were asked to present their arm with the inserted cannula to study staff through a latex sleeve in the door of the calorimeter. The latex sleeve was adjusted to fit securely around the subject's arm in order to maintain the seal of the calorimeter. Details of the samples taken and the analyses performed are discussed in 4.2.3.5. As these samples were designed to measure the effect of manipulations in P:E ratio on markers of satiety and metabolic risk these results are presented in Chapter 4.

All urine was collected whilst subjects were in the calorimeter. Two 5.5 ml aliquots of urine were stored from each sample and the volume of each sample was recorded. A 24 h collection was obtained from the second sample on day four until the first sample on day five.

Subjects completed two periods of exercise on day four, at 1500 and at 2000. Subjects cycled on the exercycle in the calorimetry room for 30 minutes at 50 watts with a pedalling speed between 40 and 60 rev per minute.

EE was measured on the night of day 3, then from the morning of day 4 subjects were given a fixed energy diet with EI based on BMR (measured by GEM) on day 1 of the study (BMR plus allowance for exercise). EE was measured until the morning of day 5.

### *5.2.2.1 Dietary Manipulation*

On day 4, isoenergetic meals of fixed energy content were provided at 0930, 1330, and 1830. The energy content of the meals was calculated for individual subjects based on BMR multiplied by 1.21 plus an allowance for two 30 min periods of exercise at 50 watts external work. BMR was measured by GEM calorimeter on day 1. Individual meals were prepared and weighed in advance, and subjects were encouraged to consume all food provided. Any remaining food was weighed and recorded. All food was of the same composition as during the *ad libitum* days 1-3 (comprising 10%, 15% or 25% of energy from protein with fat constant at 30% of energy) and used the same recipes as on day 2. Initially it was planned to provide total energy evenly distributed throughout the day, however during preliminary testing of the meals subjects experienced difficulty consuming 1/3 of the total energy for breakfast. To ensure that subjects were able to comfortably consume the total energy for the day 25% of energy was provided for breakfast, 35% for lunch (20% as the main course and 15% as the dessert), and 40% for dinner (25% as the main course and 15% as the dessert) (Table 5.2.1).

Meals were prepared for individual subjects in the kitchen at MRC HNR and transported to the CRF on the evening of day 3. Meals were either prepared specifically with recipes adjusted for the required amount of ingredients, or pre-prepared meals were used and a proportion of the meal removed to produce meals of the appropriate energy content. The

meals were the same as those provided on day 2 and details of the recipes and nutritional content are presented in Table 4.2.4 and Appendix II.1.

**Table 5.2.1 Proportion of fixed-energy intake that each dish provided for three meals**

| Meal | | Proportion of daily energy intake that each meal provided (%) |
|---|---|---|
| Breakfast | Porridge | 25 |
| Lunch | Vegetable Curry | 20 |
| | Strawberry Yoghurt | 15 |
| Dinner | Pasta | 25 |
| | Rice Pudding | 15 |

## 5.2.3 Outcome measures

### *5.2.3.1 Energy expenditure*

Energy expenditure was measured continuously from when subjects entered the calorimeter until leaving it 37 hours later. For analysis, measurements were divided into time periods of total daily EE, basal metabolic rate, EE during the night while sleeping (on the first night after *ad libitum* meals and on the second night after isoenergetic meals), and post-meal EE measured after breakfast. BMR was additionally measured using a ventilated-hood indirect calorimetry system at baseline for each study week (day 1) and after four days of the study diet (day 5).

#### *5.2.3.2 Substrate oxidation*

Oxidation of protein, fat, and carbohydrate were measured whilst in the calorimeter on day 4 of the study week. Measurements were divided into total 24h oxidation, BMR, and post-meal oxidation after the fixed-energy breakfast.

Methods for calculating EE and substrate oxidation are described in 2.4.1.

#### *5.2.3.3 Data analysis*

The primary outcome measure of total 24h EE was analysed using a random effect model for continuous normal data. Analysis was performed within-subject by making subject the only random effect. The fixed effects were the percentage of protein. The primary comparisons of interest - 25% vs. 15% protein and 10% vs. 15% protein - were estimated with 95% confidence intervals. Period-specific baseline measurements were used for analysis of BMR by ventilated-hood indirect calorimetry.

## 5.3 Results

### 5.3.1 Subject Characteristics

The study population is described in Chapter 4 and baseline characteristics are presented in Table 4.3.1.

### 5.3.2 Meals

Two subjects were unable to consume their entire daily energy provision. Both subjects felt unwell during the stay in the calorimeter and ate very little of one meal. There was no difference between the diets in the mean energy that was not consumed ($p = 0.48$) and therefore there was no significant difference in the total energy intake on each study visit (10%P 8584 ± 444, 15%P 8233 ± 444, 25%P 8350 ± 444, mean energy provided 8495 ±384 kJ).

### 5.3.3 Urinary nitrogen

Urinary nitrogen excretion, collected on day 4 of the study visits, differed significantly between the diets (Figure 5.3.1). Nitrogen excretion during the 25%P diet (0.77 ± 0.07%) was significantly higher than during the 10%P diet (0.40 ± 0.07%) ($P < 0.0001$) and the 15%P diet (0.49 ± 0.07%) ($P<0.0001$). The difference between the 10% and 15%P diets was not significant ($P = 0.184$). Individual results are shown in Figure 5.3.2. There was no effect of visit order on nitrogen excretion rates.

**Figure 5.3.1 Mean (± SEM) urine nitrogen collected on day 4 of each study visit during which subjects ate diets of different protein composition.**

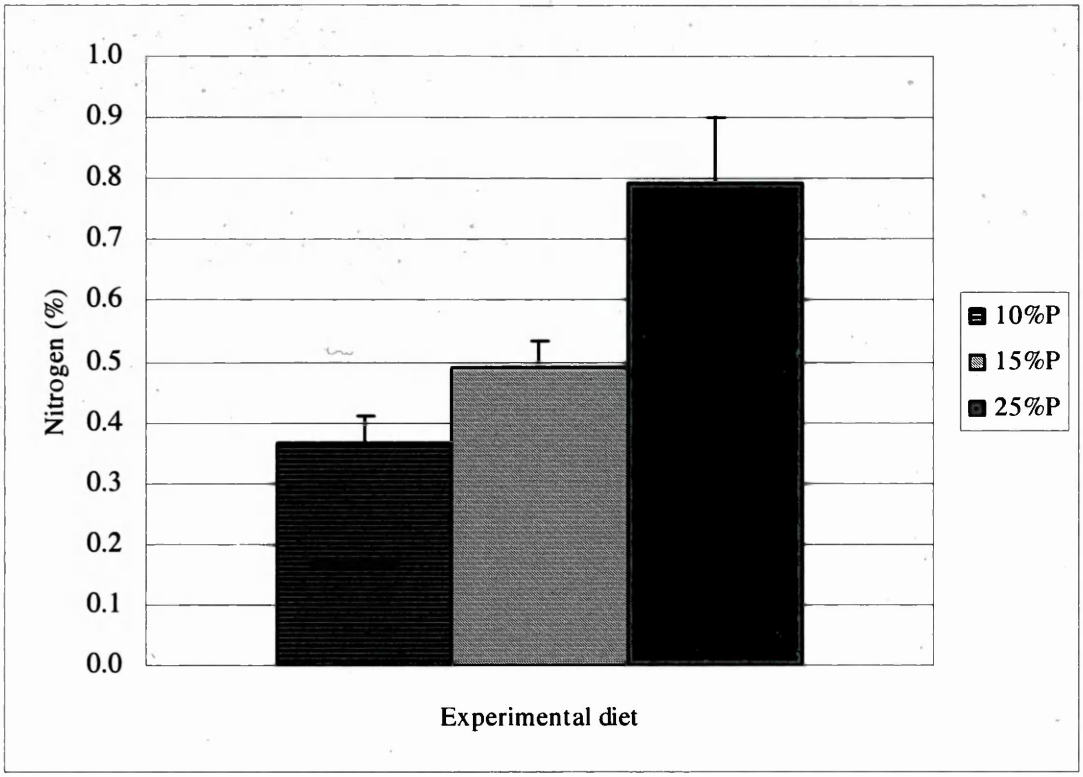
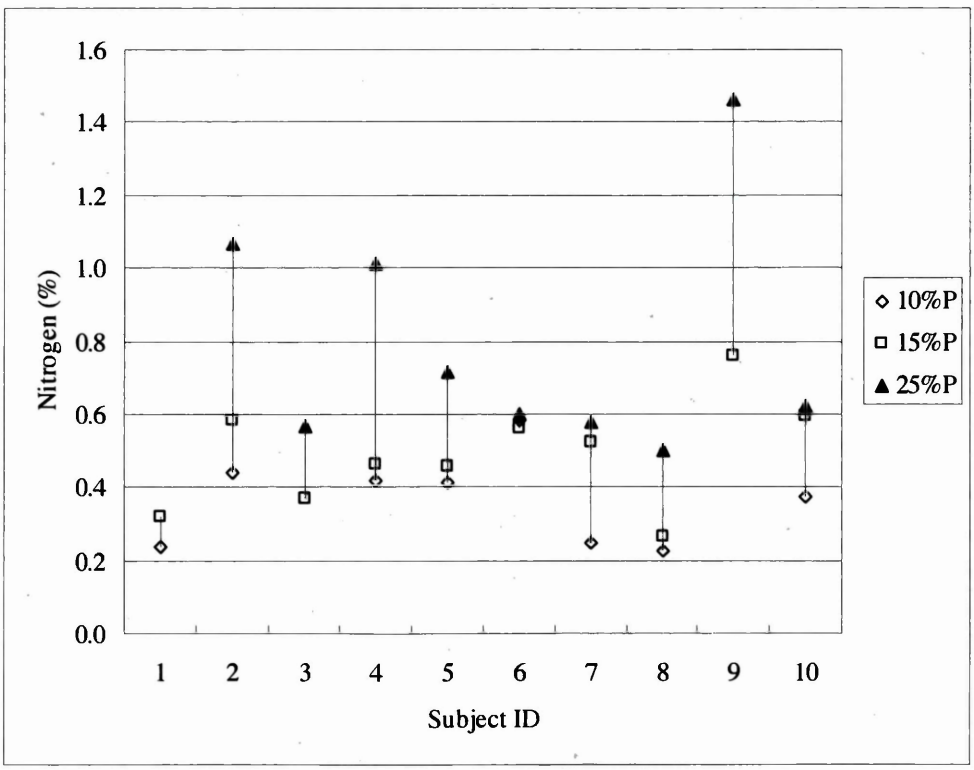



**Figure 5.3.2 Urinary nitrogen excretion on day 4 of each study visit for individual subjects.**



### 5.3.4 Outcome measures

*5.3.4.1 Energy expenditure and metabolic rate measured by whole-body calorimeter*

Measurements of total energy expenditure and basal metabolic rate recorded during the 36 h period in the calorimeter are presented in Table 5.3.1. There was no difference between dietary treatments in BMR on the first or second morning (Figure 5.3.3, Figure 5.3.4), DIT after breakfast on the first day in the calorimeter (Figure 5.3.5), SMR on the first or second night (Figure 5.3.6, Figure 5.3.7), or total energy expenditure (Figure 5.3.8).

**Table 5.3.1 Energy expenditure and metabolic rate measured by calorimeter during specific time periods for each study visit (mean ± SEM)**

| EE (kJ/min) | 10%P | 15%P | 25%P | p |
|---|---|---|---|---|
| **BMR day 1 (kJ/min)** | 4.5 ± 0.3 | 4.6 ± 0.3 | 4.4 ± 0.3 | 0.47 |
| **BMR day 2 (kJ/min)** | 4.5 ± 0.3 | 4.7 ± 0.3 | 4.6 ± 0.3 | 0.26 |
| **DIT (kJ/min)** | 6.1 ± 0.3 | 6.2 ± 0.3 | 6.3 ± 0.3 | 0.38 |
| **DIT (MJ/3 h)** | 1.1 ± 0.6 | 1.1 ± 0.6 | 1.1 ± 0.6 | 0.38 |
| **SMR night 1 (kJ/min)** | 4.2 ± 0.3 | 4.3 ± 0.3 | 4.4 ± 0.3 | 0.11 |
| **SMR night 2 (kJ/min)** | 4.1 ± 0.2 | 4.1 ± 0.2 | 4.1 ± 0.2 | 0.81 |
| **TEE (kJ/min)** | 6.0 ± 0.3 | 6.1 ± 0.3 | 6.1 ± 0.3 | 0.47 |
| **TEE (MJ/24 h)** | 8.7 ± 0.4 | 8.8 ± 0.4 | 8.8 ± 0.4 | 0.47 |

**Figure 5.3.3 Measure of BMR on the first morning in the calorimeter of each study diet for individual subjects measured by calorimeter.**

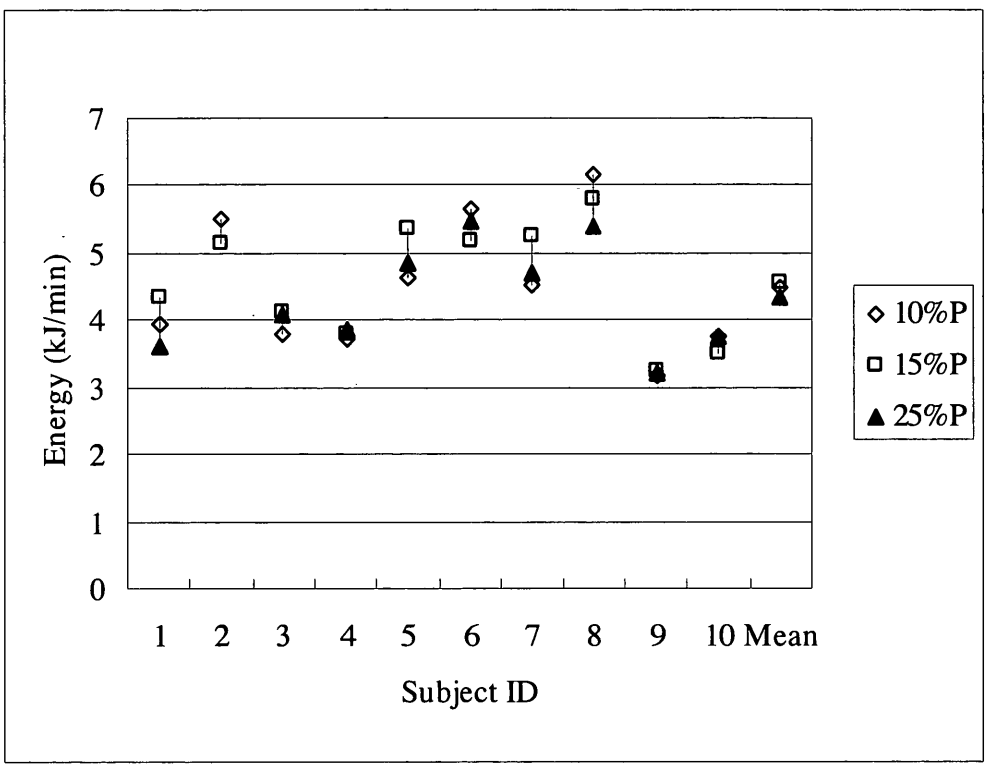



**Figure 5.3.4 Measure of BMR on the second morning of each study diet for individual subjects measured by calorimeter**

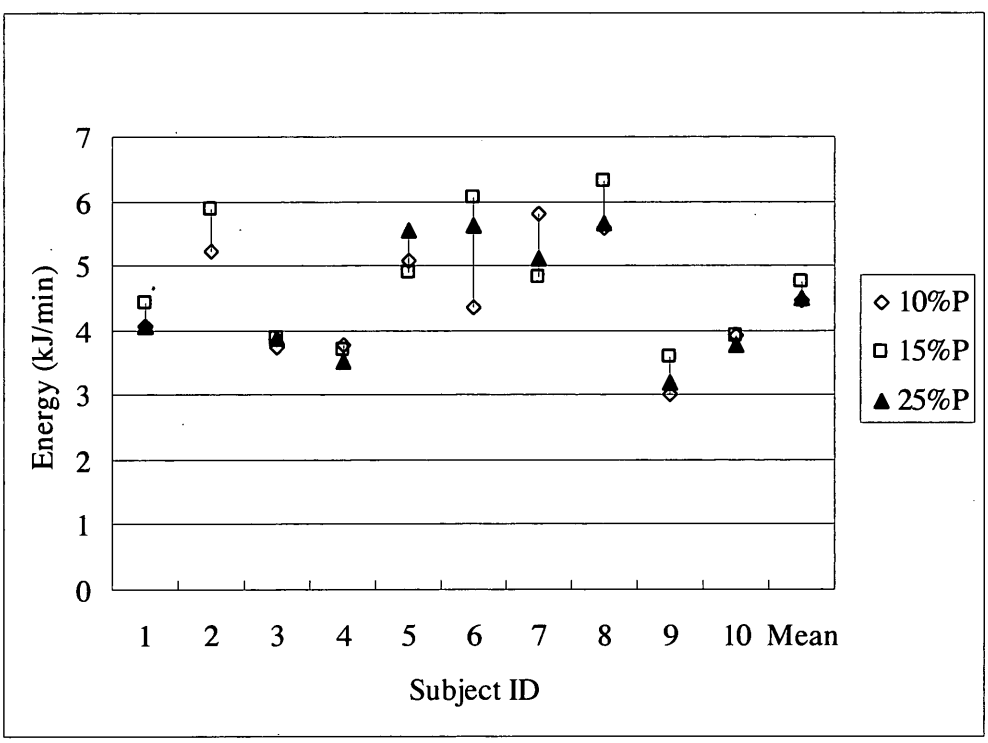


**Figure 5.3.5 DIT, measured in the calorimeter over 3 h after a fixed-energy breakfast on each study diet**

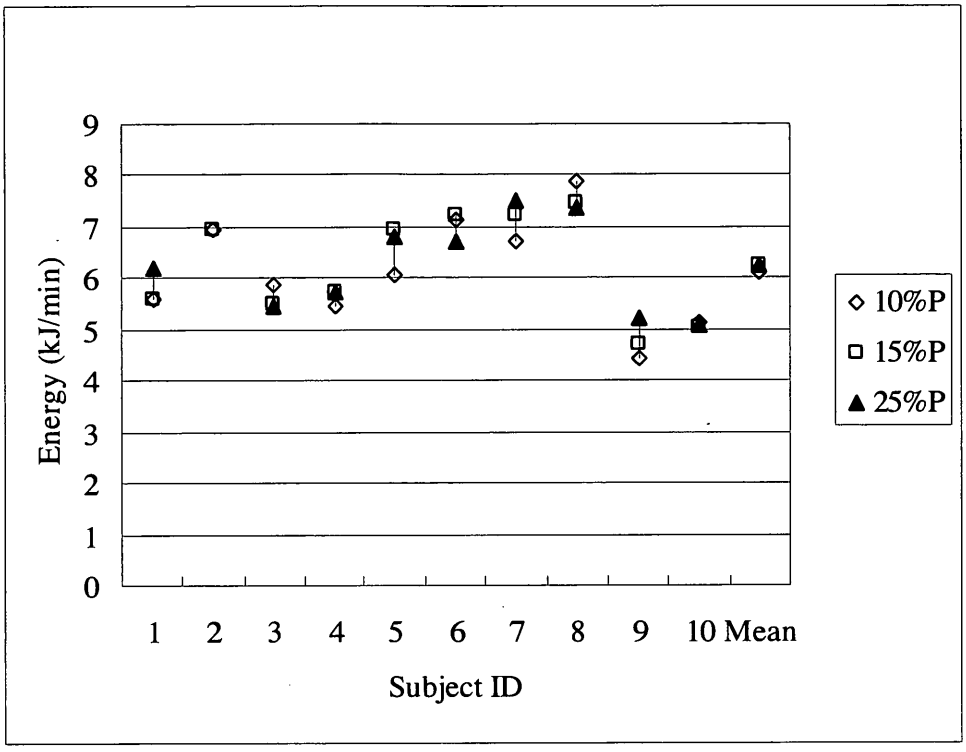



**Figure 5.3.6 Measure of SMR on night 1 of each study diet for individual subjects measured by calorimeter**

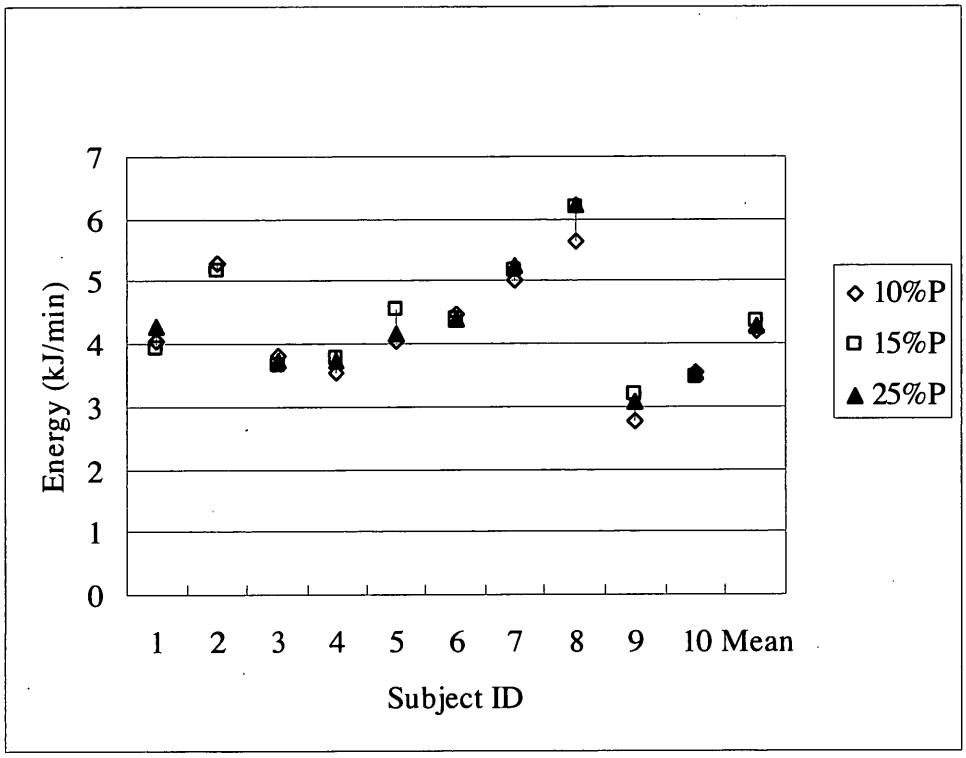


**Figure 5.3.7 Measure of SMR on night 2 of each study diet for individual subjects measured by calorimeter**

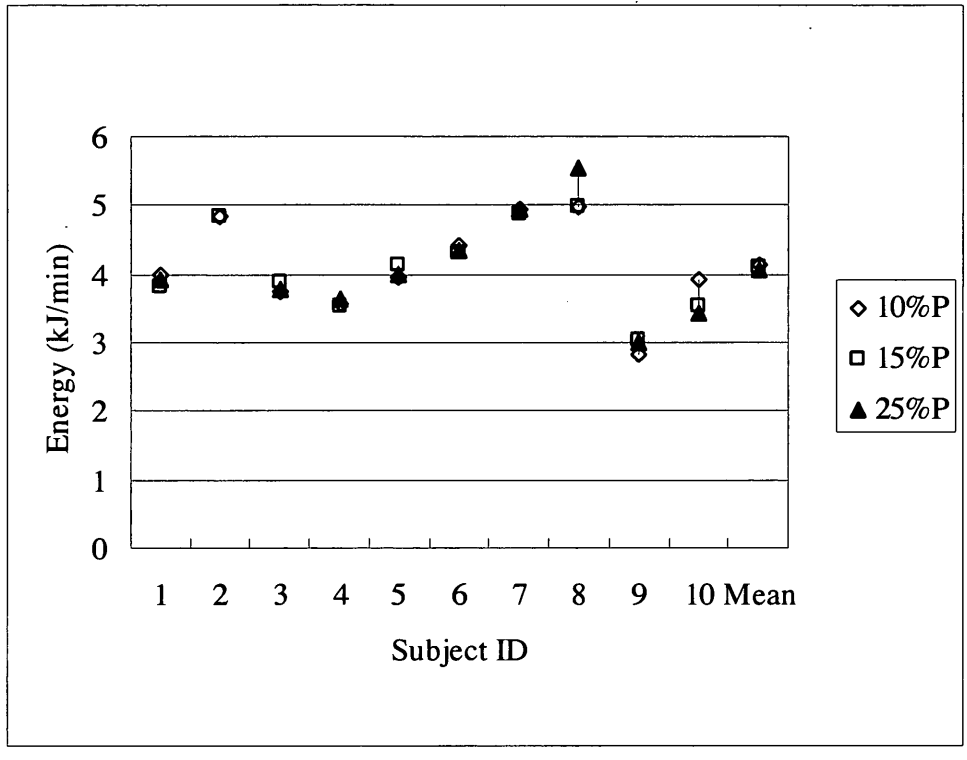
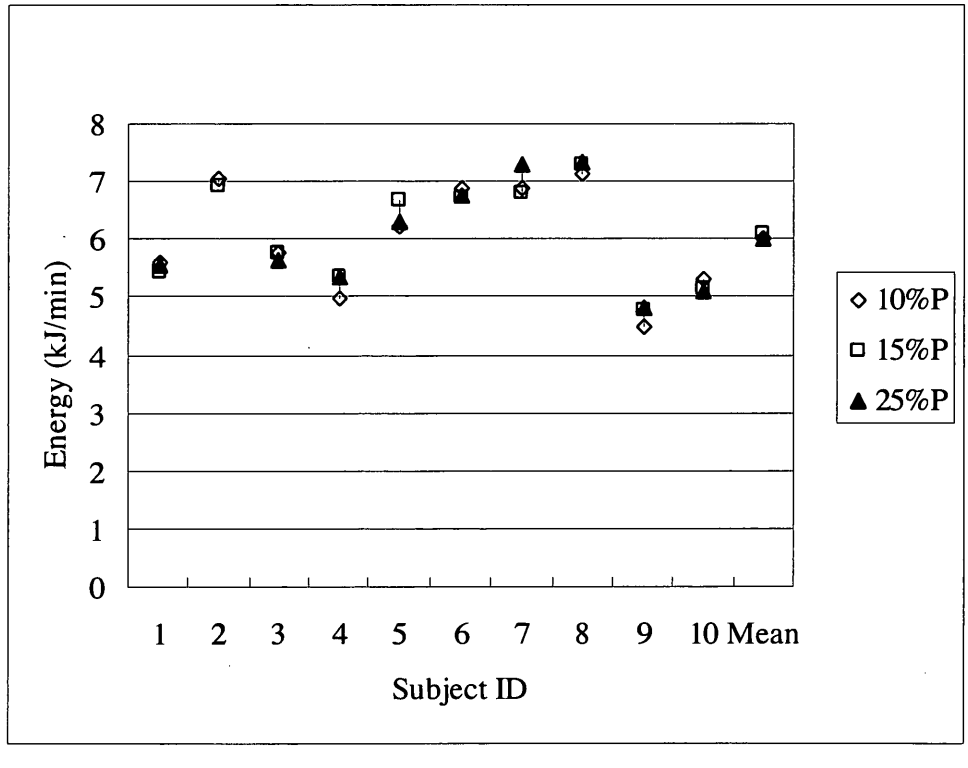



**Figure 5.3.8 Measure of TEE of each study diet for individual subjects measured by calorimeter**



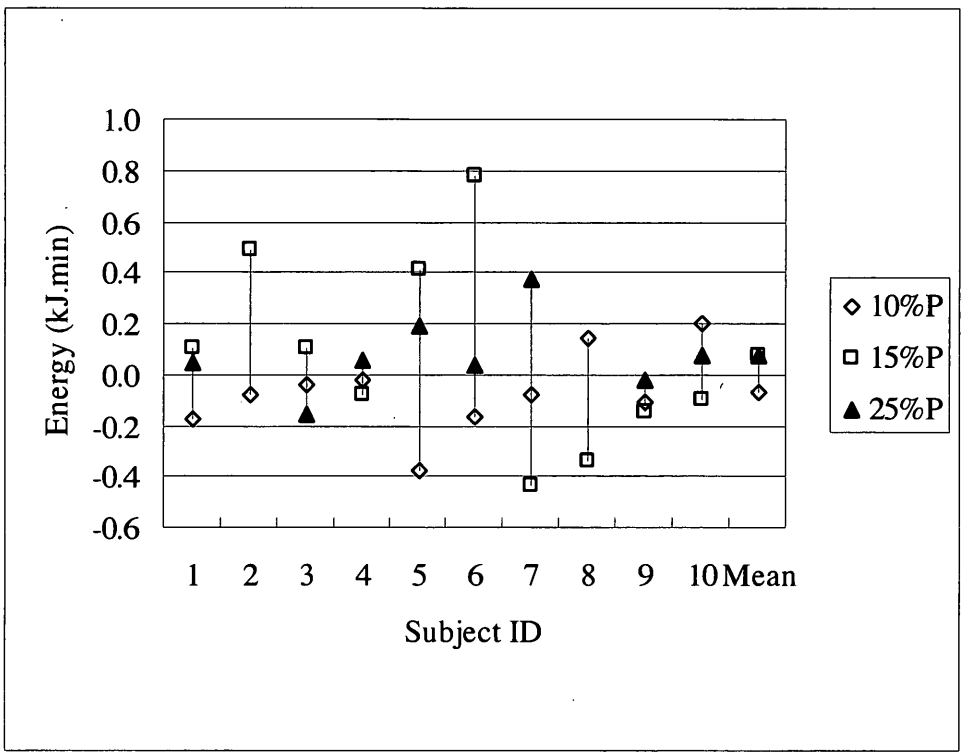
*5.3.4.2 Basal metabolic rate measured by ventilated hood (GEM)*

Resting Energy Expenditure (REE) did not differ between diet treatments on day 1 of the study week (P = 0.73) and there was no difference between day 1 and day 5 for any of the study diets (Table 5.3.2). On day 5 REE was higher after the 25%P diet (4.63 ± 0.3 kJ/min, p = 0.045) than the 10%P diet (4.50 ± 0.3 kJ/min). The mean difference between day 1 and day 5 did not differ between diets (10%P, -0.07 ± 0.42, 15%P, 0.08 ± 0.42, -0.98 ± 0.42) (P = 0.13). Individual differences in REE between day 1 and day 5 are presented in Table 5.3.3.

**Table 5.3.2 Mean (SEM) REE measured by GEM on day 1 and 5 of each diet**

| Diet | REE (kJ/min) | | |
|---|---|---|---|
| | D1 | D5 | p |
| 10%P | 4.57 ± 0.2 | 4.50 ± 0.3 | 0.16 |
| 15%P | 4.48 ± 0.2 | 4.55 ± 0.3 | 0.53 |
| 25%P | 4.53 ± 0.2 | 4.63 ± 0.3 | 0.19 |
| p | 0.73 | 0.13 | |

**Figure 5.3.9 Change in REE from day 1 to day 5 of each study diet measured by GEM for individual subjects**




*5.3.4.3 Substrate oxidation measured by whole-body calorimeter*

Oxidation of protein, fat and carbohydrate during the time spent in the calorimeter is presented in Table 5.3.3 separated into BMR, DIT and TEE.

During the one hour measurement of BMR on the first morning in the calorimeter (after three days of *ad libitum* consumption of the study diet) protein oxidation was higher with the 25%P diet than the 15%P ($p = 0.014$) and 10%P diets ($p = 0.001$), but there was no difference in protein oxidation between the 15%P and the 10%P diets ($p = 0.43$). Fat oxidation was higher with the 25%P diet ($p = 0.035$) than the 10%P, but there was no evidence of a difference between the 25%P and the 15%P diets and the 15%P compared to the 10%P diet. Carbohydrate oxidation was lower during the 25%P diet compared to the

10%P ($p < 0.0001$) and 15%P diets ($p = 0.02$). There was no difference between the 15%P and the 10%P diets (Figure 5.3.10).

**Table 5.3.3 Oxidation of protein, fat and carbohydrate measured in kJ for each study visit, during specific time periods in the calorimeter (mean ± SEM).**

* 25%P diet different from 10%P, § 25%P diet different from 15%P, # 15%P diet different from 10%P

| Energy Expenditure | Diet | Protein | Fat | Carbohydrate |
|---|---|---|---|---|
| **BMR day 1 kJ/h** | 10 %P | 52.4 ± 8.3 | 76.8 ±20 | 140.0 ± 26 |
| | 15 %P | 59.2 ± 8.3 | 101.0 ± 20 | 114.0 ± 26 |
| | 25 %P | 81.2 ± 8.6 | 115.7 ± 21 | 67.0 ± 27 |
| **p** | | 0.001*§ | 0.035* | *§ |
| **BMR day 2 kJ/h** | 10 %P | 38.6 ± 4.6 | 113.0 ± 18 | 115.5 ± 20 |
| | 15 %P | 56.3 ± 4.6 | 150.0 ± 18 | 78.6 ± 20 |
| | 25 %P | 87.7 ± 4.8 | 134.2 ± 19 | 50 ± 21 |
| **p** | | <0.0001*§# | 0.05 | 0.004* |
| **DIT MJ/3 h** | 10 %P | 0.16 ± 0.02 | 0.31 ± 0.05 | 0.63 ± 0.07 |
| | 15 %P | 0.22 ± 0.02 | 0.34 ±0.05 | 0.57 ± 0.07 |
| | 25 %P | 0.24 ± 0.02 | 0.39 ± 0.05 | 0.50 ± 0.07 |
| **p** | | <0.001*# | 0.06 | 0.002* |
| **TEE MJ/24 h** | 10 %P | 1.14 ± 0.08 | 2.85 ± 0.3 | 4.69 ± 0.47 |
| | 15 %P | 1.56 ± 0.08 | 2.87 ± 0.3 | 4.32 ±0.47 |
| | 25 %P | 2.22 ± 0.08 | 3.27 ± 0.3 | 3.32 ± 0.47 |
| **p** | | <0.001*§# | 0.028*§ | <0.001*§ |

On the second day in the calorimeter during the measurement of BMR (after one day of fixed-energy intake of the study diet), there was a dose-response increase in protein oxidation with an increase in consumption, as illustrated in Figure 5.3.11. Protein oxidation during the 25%P diet was higher than during the 15%P ($p < 0.0001$) or the 10%P diets ($p < 0.0001$), and was higher during the 15%P than 10%P diet ($p = 0.006$). There was no evidence of a difference in fat oxidation between any of the study diets. Carbohydrate oxidation was lower during the 25%P than the 10%P diet ($p = 0.004$), however there was no difference between the 15%P and the 10%P diets or the 25%P and the 15%P diets.

DIT was measured on each dietary treatment, over a three-hour period after a fixed-energy breakfast. Protein oxidation was higher during the 25%P than the 10%P diet ($p < 0.001$) and during the 15%P than 10%P diet ($p = 0.001$). There was no evidence of a difference between the 25%P and the 15%P diets. No difference in fat oxidation was evident between the study diets. Carbohydrate oxidation was lower during the 25%P than the 10%P diet ($p = 0.002$) but there was no difference between any of the other study diets (Figure 5.3.12).

Substrate oxidation during the 24 h measurement of TEE is presented in Figure 5.3.13. Protein oxidation was higher during the 25%P than the 15%P ($p < 0.001$) and the 10%P diets ($p < 0.0001$), and the 15%P diet produced greater protein oxidation than the 10%P diet ($p <0.0001$). Similarly fat oxidation was higher during the 25%P diet compared to the 15%P ($p = 0.038$) and 10%P diets ($p = 0.028$), but there was no difference between the 15%P and the 10%P diets. Carbohydrate oxidation was lower during the 25%P than the 15%P ($p < 0.001$) or 10%P diets ($p < 0.001$). There was no evidence of a difference between the 15%P and the 10%P diet ($p = 0.078$).

**Figure 5.3.10 Mean ± SEM substrate oxidation for all subjects during measurement of BMR on day 1 in the calorimeter**

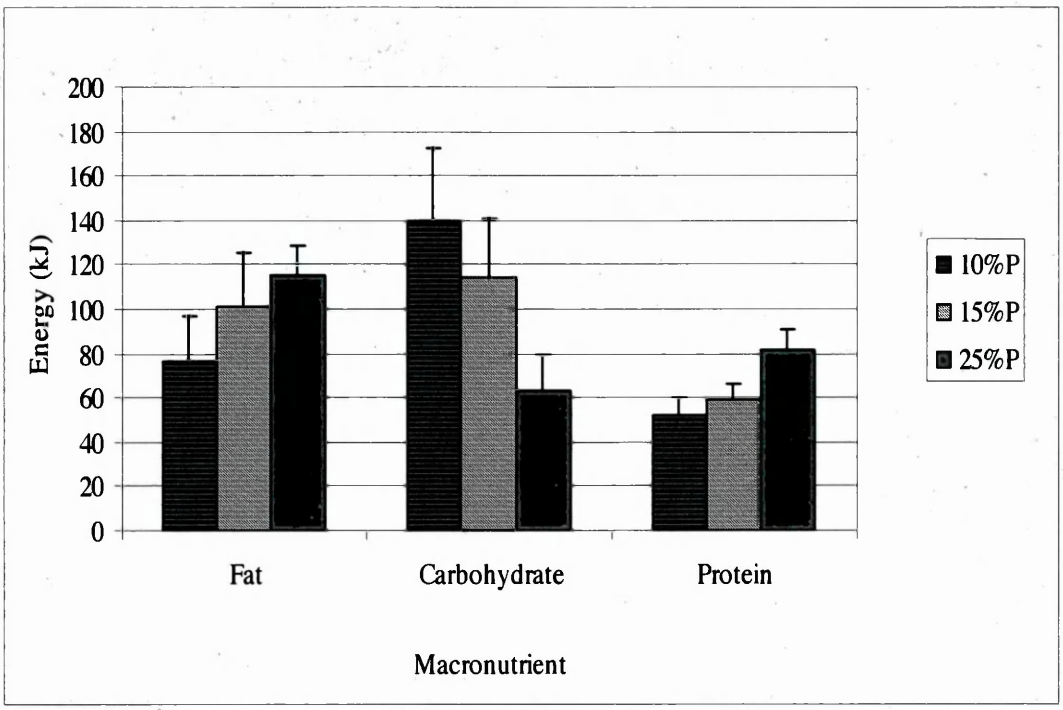



**Figure 5.3.11 Mean ± SEM substrate oxidation for all subjects during measurement of BMR on day 2 in the calorimeter**

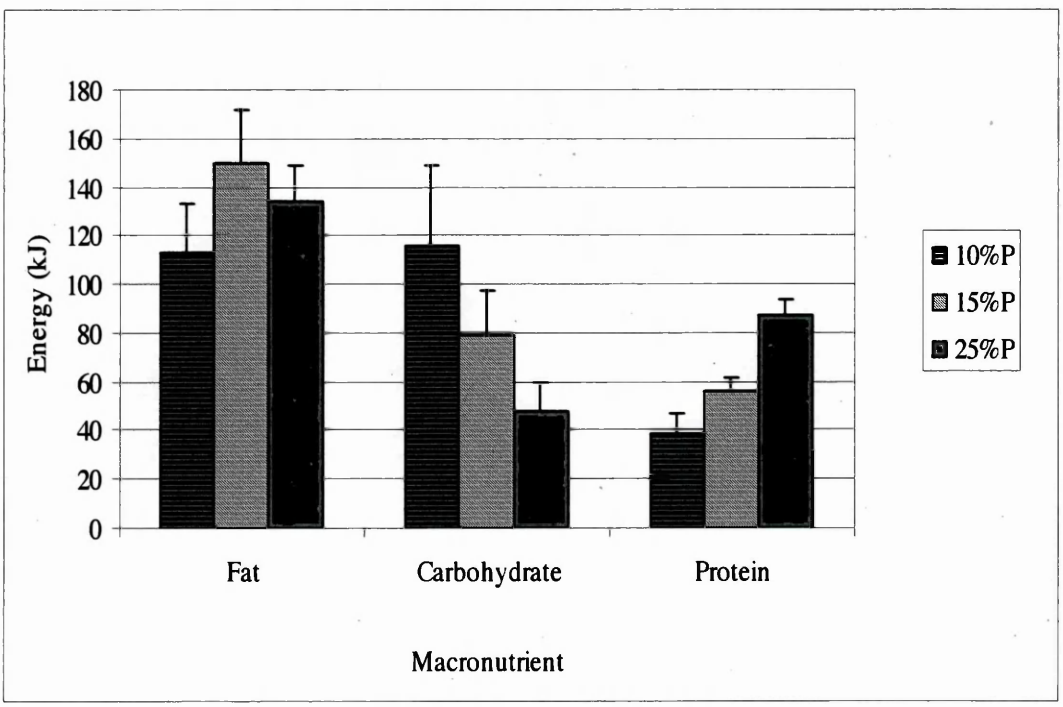


**Figure 5.3.12 Mean ± SEM substrate oxidation for all subjects after breakfast measured in the calorimeter**

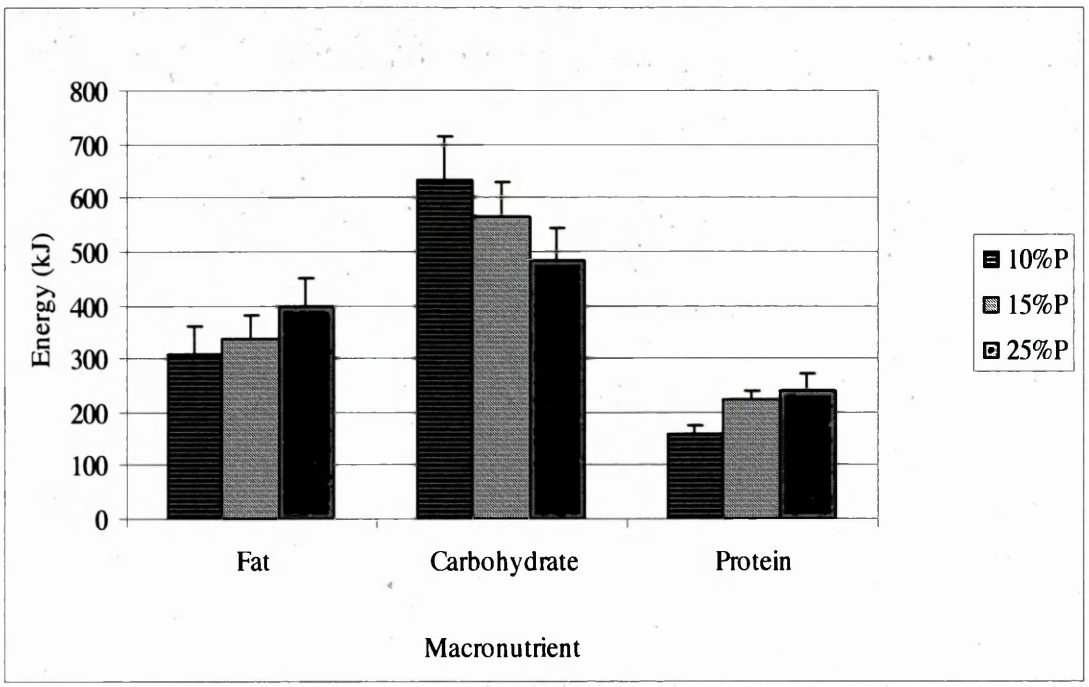
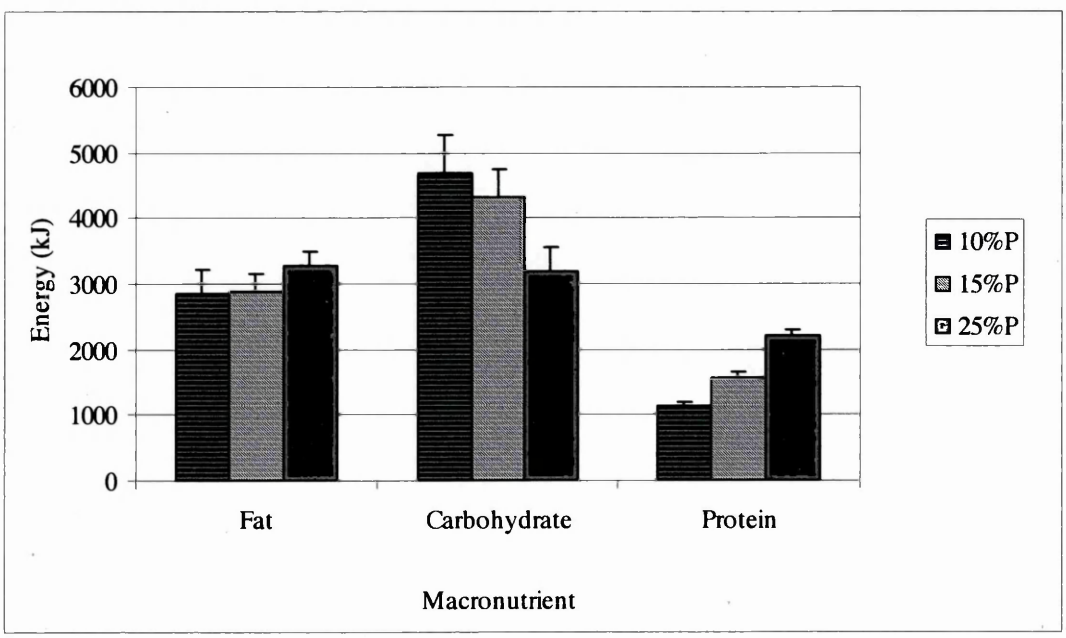



**Figure 5.3.13 Mean ± SEM substrate oxidation for all subjects during the 24 h period in the calorimeter**



Oxidation of fat, carbohydrate and protein was compared to intake for each substrate to calculate substrate balance. There was no difference between intake and oxidation of fat during the 10%P diet or 15%P diet, but fat oxidized during the 25%P diet (3286 ± 185 kJ) was significantly higher than fat intake (2504 ± 176 kJ) ($p = 0.001$) (Figure 5.3.14). Oxidation of carbohydrate did not differ from intake during the 10%P or 15%P diets but was significantly lower than intake during the 25%P diet (intake 3757 ± 289 kJ, oxidation 3243 ± 298 kJ, $p = 0.033$) (Figure 5.3.15). Protein oxidation was higher than intake during the 10%P diet (intake 858 ± 53 kJ, oxidation 1140 ± 53 kJ, $p < 0.0001$) and the 15%P diet (intake 1235 ± 76 kJ, oxidation 1560 ± 76 kJ, $p < 0.0001$), but not during the 25%P diet (intake 2087 ± 105 kJ, oxidation 2231 ± 109 kJ, $p = 0.163$) (Figure 5.3.16). Total 24 h energy oxidized did not differ from intake during the 10%P diet (intake 8584 ± 465 kJ, oxidation 8682 ± 465 kJ, $p = 0.163$) but oxidation was higher than intake during the 15%P diet (intake 8233 ± 408 kJ, oxidation 8758 ± 408 kJ, $p < 0.0001$) and the 25%P diet (intake 8350 ± 428 kJ, oxidation 8835 ± 432 kJ, $p = 0.02$) (Figure 5.3.17).

**Figure 5.3.14 Mean ± SEM 24 h intake and oxidation of fat for all subjects during each study diet, plus calculated fat balance**

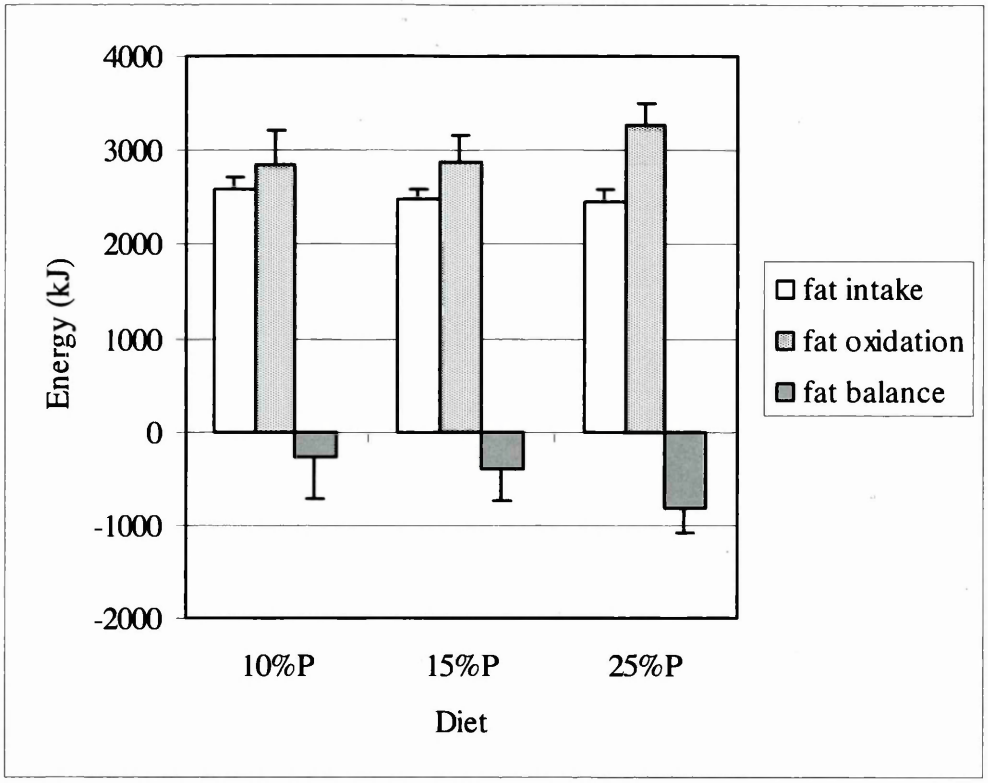



**Figure 5.3.15 Mean ± SEM 24 h intake and oxidation of carbohydrate for all subjects during each study diet, plus calculated carbohydrate balance**

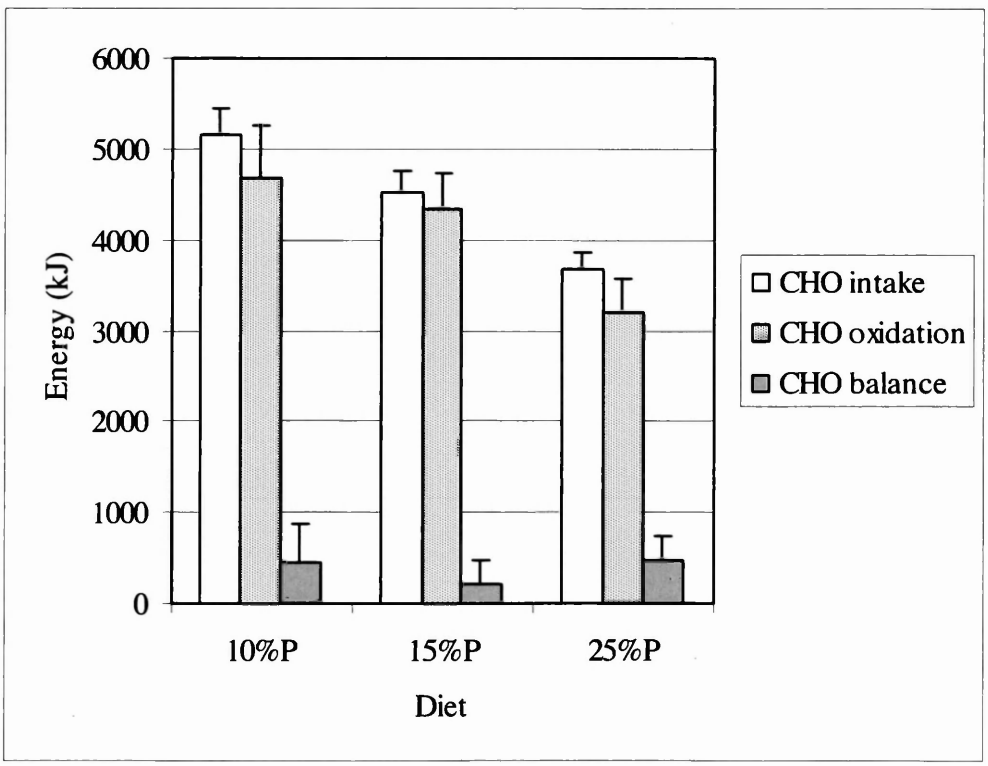


**Figure 5.3.16 Mean ± SEM 24 h intake and oxidation of protein for all subjects during each study diet, plus calculated protein balance**

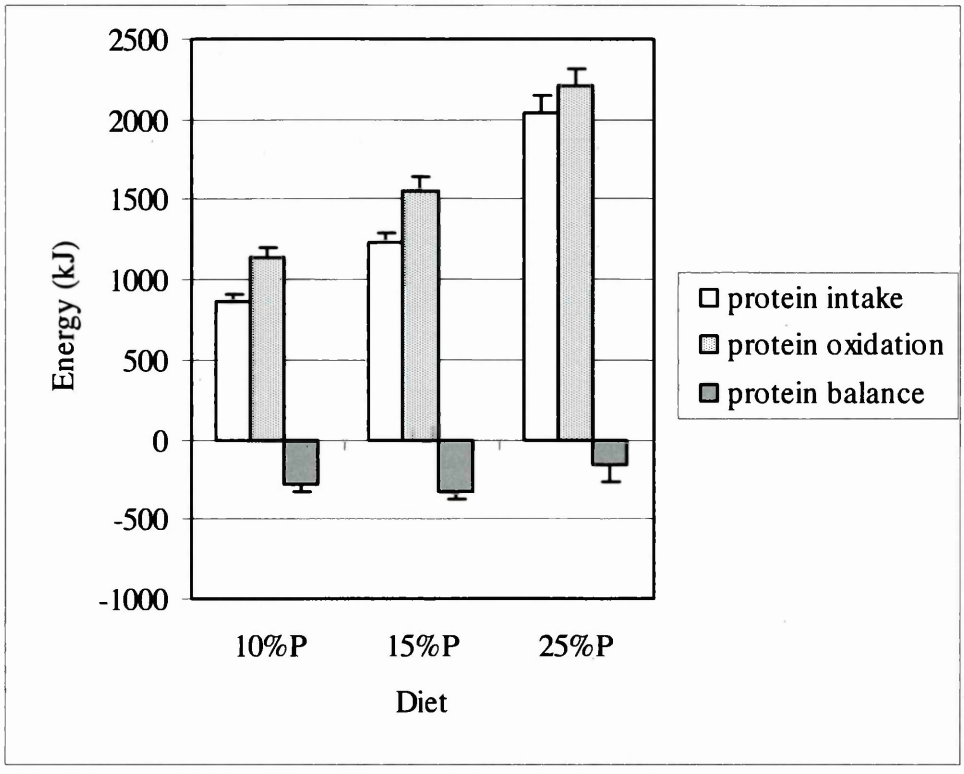
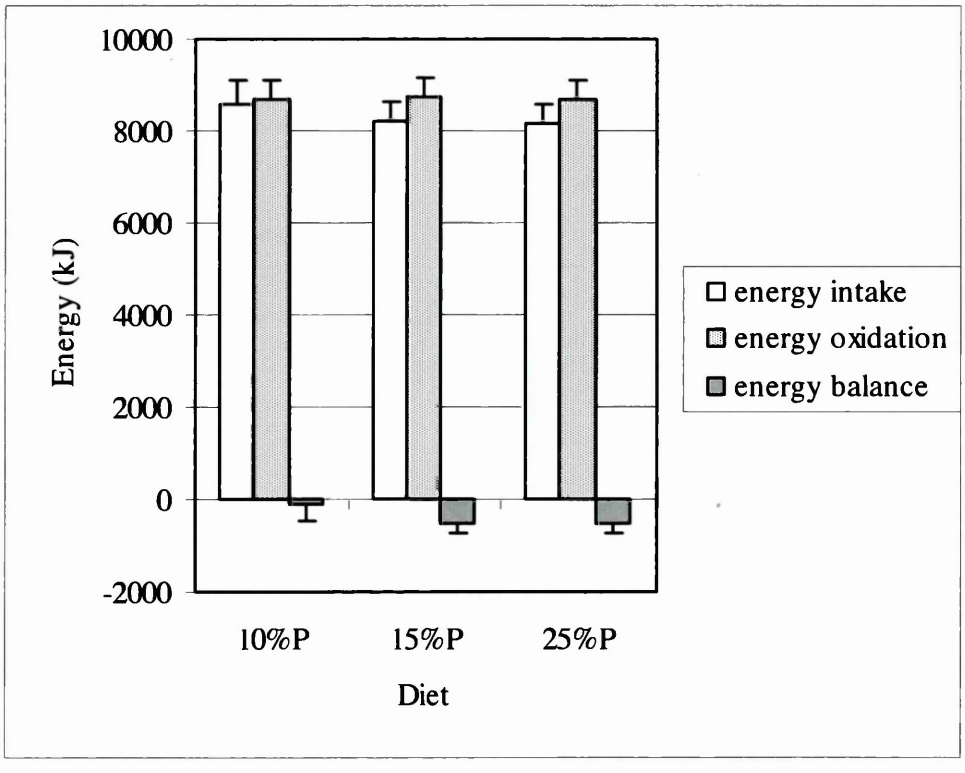



**Figure 5.3.17 Total 24 h energy intake and oxidation during each study diet**



## 5.4 Discussion

This chapter reports the findings of a crossover study to investigate the effect of isoenergetic diets differing in their protein:energy ratio on energy expenditure. A major strength of this study was that experimental diets had been consumed for three days prior to the measurements of energy expenditure and substrate oxidation to allow time for adaptation. Additionally the intervention diets were designed to be representative of habitual diets, with the percentage of energy provided from protein within the acceptable limits of normal intake and the meals composed of commonly eaten dishes. In contrast to previous studies with more extreme dietary manipulations the findings from this study may be more easily translated to diets in the general population. Measurements were made in a whole body calorimeter and the conditions during the investigation periods were tightly standardised, to allow for consistent within-subject comparisons and increased power to detect any effects.

The findings of this study reveal that total energy expenditure is not affected by manipulating the P:E ratio of the diet over a four-day period. Measurements of substrate oxidation suggested that protein and carbohydrate oxidation follows or matches intake, but fat oxidation increases over intake when the diet is high in protein and lower in carbohydrate, leading to a small negative energy balance.

### 5.4.1 Effect of diets of varying P:E ratios on energy expenditure

This study demonstrated that energy expenditure does not differ between diets of varying P:E ratio when lean, healthy subjects are provided with isoenergetic diets over 24 h, while undertaking identical activities in a room calorimeter. Despite three days of adaptation to the study diets, during which all food was consumed *ad libitum*, there was no difference

between diets composed of 10%, 15% or 25% of energy from protein on total energy expenditure, DIT, SMR or BMR. Likewise there was no difference in the change in BMR between the first day of each study week and the morning after the isoenergetic diet in the calorimeter.

As discussed in Chapter 4 (4.2.2.3) the meals were carefully designed and prepared to closely match individual energy requirements for each study visit with a different P:E ratio. The dishes were familiar to the subjects and were all made from standard household ingredients. Subjects were therefore readily able to consume their total energy provision without difficulty in all but two instances. On these two occasions subjects felt unwell whilst in the calorimeter and were unable to consume their entire meal. The difference in energy intake between these and dietary treatments was not significant (Section 5.3.2) and both subjects were able to complete their calorimeter stay and the remainder of the study. The difference in composition between the study diets was not large. The lower and higher proportions of total energy from protein in this study (10%P vs. 25%P) are both within the normal limits of recommended protein consumption as the study was specifically designed to investigate whether small changes in the proportion of energy from protein, which could be readily adopted by the general population, might affect energy expenditure. As illustrated by urine nitrogen excretion (Figure 5.3.1) there was a clear difference between nitrogen excretion, and therefore protein intake, on each of the study visits, confirming that food intake accurately reflected the planned diets.

While there has been uncertainty regarding the effect of protein on total energy expenditure, it has generally been believed that higher protein intake increases DIT. When 12 women were provided with isoenergetic 4-day diets containing 10% or 30% of energy from protein, DIT was higher during the high-protein diet (0.91 MJ/d) than the low-

protein diet (0.69 MJ/d), and there was a trend to higher 24h EE (Lejeune *et al.*, 2006). Elevated DIT was also demonstrated in 36 subjects who consumed a high- or low-protein (32% or 10% of energy from protein) test meal as part of an energy restricted weight loss diet (Luscombe *et al.*, 2003). DIT was 2% greater after the high-protein than the low-protein meal at baseline, but there was no difference in DIT after 16 weeks of energy restriction. It is possible that the higher proportion of energy from protein (above the recommended upper limit) in these studies, and the subsequent increase in protein metabolism, was sufficient to increase post-prandial EE. In the latter study TEE and RMR were measured at baseline and after 16 weeks of the study diet and did not differ between diets. There was however less of a difference between the diets during the 16 week intervention period (high-protein diet comprised 30% of energy as protein and the low-protein diet 15% of energy as protein) than on the test day (32% versus 10%). The small difference in DIT was not sufficient to explain the greater weight loss observed in the high-protein group over the whole intervention period.

The lack of an effect of diets varying in protein content on DIT demonstrated in this study is supported by Smeets *et al*, 2008 (Smeets *et al.*, 2008), who provided a test lunch to 30 subjects containing 10% or 25% of energy from protein, and comprising 35% of each subject's daily energy requirements. They observed no difference in EE after the test lunch between the high and low-protein meals measured via a ventilated hood (although there was a non-significant increase in EE after the high-protein lunch). Similarly Raben *et al*, 2003 (Raben *et al.*, 2003) found no difference in EE measured over five hours in 19 subjects after test meals containing 32% or 12% of energy from protein and 24% of energy requirements.

One reason to explain why previous studies have suggested protein promotes DIT and leads to increased 24h EE compared with carbohydrate and fat is that the meals provided were more extreme than those used in this study. The thermic response to hourly meals containing 70% of energy from protein or 70% from carbohydrate found energy expenditure over nine hours greater after the high-protein meals (9.6% of energy intake) than after the carbohydrate meals (5.7%) (Robinson *et al.*, 1990). These meals were prepared with protein powder and liquid formula and therefore could not be sustained in the long term.

These studies suggest that to demonstrate increased post-prandial energy expenditure after a high-protein meal the proportion of protein in the meal must be elevated above the recommended limit, and even in these circumstances greater DIT is insufficient to lead to a net increase in TEE.

Another factor that may have influenced EE in this study was that the proportion of total daily energy intake provided at the test meal was only 25%, with a mean of 2.1 ± 0.1 MJ. While we intended to spread energy intake evenly throughout the day in the calorimeter, subjects had difficulty consuming the larger breakfasts. Prior to the study commencing the breakfast meal was reduced in size to maximise complete consumption of each meal hence mean difference in protein intake was only 12 g to 30 g at this meal.

One observation suggests that protein type may additionally influence energy expenditure. 12 subjects consumed diets containing 29% of energy from protein, predominantly from pork or soy, or 11% of energy from protein. 24 h EE was higher with the pork diet than with the soy or carbohydrate diets (Mikkelsen *et al.*, 2000). Limiting the meals to one protein type may therefore have been expected to produce a difference in EE between the

study diets. However, in the current study the meals were purposefully designed to be of mixed protein source, to replicate a standard diet as much as possible.

### 5.4.2 Effect of diets of varying P:E ratios on substrate oxidation

Although EE did not differ between diets, this study demonstrates that the P:E ratio of the diet affects substrate oxidation. Protein and carbohydrate oxidation followed the same pattern as dietary intake suggesting autoregulation of substrate oxidation. The 24 h measurement demonstrated that fat oxidation was significantly higher during the 25%P diet than the 15%P or 10%P diets despite the proportion of fat in each diet being the same (30% of energy intake). Consequently, significantly more fat was oxidized during the 24 h period than was consumed (a difference of 782 kJ/24 h). Oxidation of carbohydrate was lower than intake during the 25%P diet; with a positive balance of 514 kJ/24 h. Total 24 h oxidation was therefore higher than intake during the 25%P protein diet.

The differences in oxidation observed are supported by a study in 12 women who consumed meals containing 10% or 30% of energy from protein and 30% from fat over 24 h. During the high-protein diet subjects were in positive protein balance and negative fat balance, while during the low-protein diet subjects were in positive carbohydrate balance and additionally were in a small positive balance for protein and fat (Lejeune *et al.*, 2006). In a further study, 26 women consumed meals either high in carbohydrate (15% of energy from protein) or high in protein (30% of energy) with fat intake constant at 30% of energy. Post-prandial oxidation measured over 6 h demonstrated greater fat oxidation after the high protein meal with no changes in carbohydrate oxidation or DIT (Labayen *et al.*, 2004). In a similar manner to the present study, there was no correlation between oxidative balance and measurement of energy expenditure, despite fat oxidation being

greater than intake, which should lead to increased EE. This data suggests that changes in substrate oxidation may be identified with small changes in diet and perhaps with less extreme manipulations than is required to measure a difference in EE.

In contrast to the studies discussed above demonstrating greater fat oxidation with increasing protein, a study using diets of similar macronutrient composition to the present study, reported that substrate oxidation did not differ after a single test meal which provided 10% or 25% of energy from protein and 30 % of energy from fat (Smeets *et al.*, 2008). A period of 2 – 4 days of adaptation is required to establish an oxidative balance when intake of substrate changes (Jebb *et al.*, 1993) (Westerterp *et al.*, 1999). As the subjects in our study had consumed the study diet *ad libitum* for three days prior to their investigation period, the measurement of their substrate oxidation more accurately reflects changes in oxidation due to the study diets.

The increase in fat oxidation with the 25%P diet is most probably due to the reduction in carbohydrate intake. Jebb *et al* (1996) demonstrated the importance of carbohydrate in the oxidative hierarchy during studies of over- and under-feeding (Jebb *et al.*, 1996). When dietary energy was present in excess, carbohydrate utilisation rates increased rapidly to match intake within a few days, while counterregulatory responses led to suppression of fat oxidation despite a substantial increase in fat intake. The converse occurred during underfeeding. While protein oxidation increased or decreased in response to intake, changes were small and balance between intake and oxidation was not achieved. There did not appear to be any autoregulation of fat oxidation, which was dependent on changes in carbohydrate and protein oxidation, predominantly that of carbohydrate.

In our study, performed during energy balance, carbohydrate oxidation followed intake as expected but protein oxidation also appeared to be able to change in response to intake. A 140% difference in protein intake between the low and high-protein diets produced a 96% difference in oxidation and there was no difference between intake and oxidation for the 25%P diet, suggesting that some autoregulation of protein oxidation did occur. It is possible that during a state of energy balance, manipulation of the macronutrient composition of the diet may stimulate protein-induced regulation of oxidation.

There is evidence that cats are able to regulate protein oxidation dependent on intake, provided the minimum protein requirement is met (Green *et al.*, 2008). In six women substrate oxidation was investigated by providing subjects with meals containing either 3.9% or 11.4% of energy from protein, 35.4% or 27.8% of energy from carbohydrate and 60% of energy from fat (Petzke & Klaus, 2008). During consumption of the low-protein diet exogenous post-prandial fat oxidation was significantly higher than during the adequate protein diet but total fat oxidation did not differ between the two, suggesting that a minimum intake may also be required in humans for autoregulation to occur.

Absolute fat intake did not differ between the diets but there was a substantially larger oxidation of fat during the 25%P diet – 782kJ during the 24 h period. The negative fat balance seen in the 25%P diet is likely to promote fat loss in the long term, and to have subsequent beneficial effects of a more favourable body composition for metabolic risk factors (as discussed in Section 1.7). Indeed, subjects with higher protein intake during weight maintenance after weight loss have a lower fat mass than subjects with lower protein intake (Westerterp-Plantenga *et al.*, 2004; Lejeune *et al.*, 2005). It appears that the ability to store dietary fat is critical to an individual's susceptibility to increase body mass

(Westerterp, 2009) and therefore any mechanism which promotes fat oxidation is advantageous to maintain or reduce body mass.

The importance of carbohydrate oxidation on food intake has been investigated in 112 subjects, predominantly Pima Indians (Pannacciulli *et al.*, 2007). Carbohydrate oxidation, measured in a calorimeter during energy balance, was a strong predictor of subsequent *ad libitum* food intake and short-term changes in body weight. The theoretical basis for this observation is that higher carbohydrate oxidation may deplete glycogen stores more readily and lead to an increase in hunger experienced, and subsequently greater food intake. Reducing carbohydrate intake may therefore reduce carbohydrate oxidation and subsequent hunger, leading to beneficial effects on body weight.

### 5.4.3 Summary

This study has shown that adjusting the P:E ratio of the diet from 10% to 25% of energy from protein, while maintaining fat at 30%, does not alter total energy expenditure, or any of the components of energy expenditure, when familiar meals are provided in energy balance in a calorimeter, after three days of *ad libitum* consumption of the study diet.

Additionally, these findings do not support previous evidence that increasing dietary protein increases DIT, although the effect on total energy expenditure has previously been less certain. Strengths of this study to support the data are that the investigations were performed in highly controlled conditions and yet the meals provided and the macronutrient manipulations were designed to be as close to habitual food consumption as possible. Additionally, three days of adaptation to each study diet was provided prior to investigation days. Providing less extreme manipulations of the diet may have reduced an

effect on DIT, which may explain the lack of consistency with previous studies which have demonstrated an increase in DIT with a high protein diet, however this study was specifically designed to utilise diets that may be able to be continued long-term.

In contrast, this study demonstrates that even relatively small manipulations in the P:E ratio of the diet alter substrate oxidation. Autoregulation of carbohydrate oxidation was evident and to a lesser extent for protein. Despite fat intake being equal in all three diets, fat oxidation was higher in the 25%P diet than the lower protein diets, and fat oxidation was higher than intake leading to a negative fat balance during the higher-protein diet, which may contribute to improvements in body composition.

The higher fat oxidation is probably due to lower intake of carbohydrate in the high-protein diet, whereby a reduction in carbohydrate oxidation produces a reciprocal increase in fat oxidation. The importance of carbohydrate oxidation in controlling hunger and food intake is also of interest when macronutrient intake is manipulated and may be important for long-term energy balance. Therefore increasing dietary protein in exchange for carbohydrate may not only reduce hunger by reducing carbohydrate oxidation. There may also be an increase in fat oxidation and reduction in fat storage, leading to long-term reductions in weight gain.

# 6 Chapter 6 Discussion

## 6.1 The need for strategies to reduce energy imbalance

In describing the normal mechanisms that regulate energy balance, the introductory Chapter 1 of this thesis demonstrates that, while complex physiological processes maintain energy balance, these biological systems may be overridden by environmental and psychological factors and lead to under- or overnutrition. The worldwide increase in obesity is caused by energy imbalance and is leading to a rapid rise in morbidity and mortality from metabolic diseases; particularly type 2 diabetes and cardiovascular disease. Furthermore it was argued that there is a pressing need to identify targets in the energy balance equation that can lead to acceptable strategies to prevent the development of obesity in the general population and promote weight loss in those currently obese, and additionally to attempt to reduce risk factors for metabolic diseases.

Dietary strategies are the foundation of any approach to alterations in energy balance because of their widespread applicability, and have been extensively studied to develop guidelines for the management of energy imbalance, particularly obesity. The increasing prevalence of popular diets has additionally led researchers to investigate the success and underlying mechanisms of novel regimes for weight loss. In part because of the widespread popularity of diets high in protein and the recognition that high-protein meals enhance satiety, protein has been recently recognised as a potentially important component in dietary strategies, where previously the macronutrients that comprise a much larger proportion of a standard diet (carbohydrate and fat) have received more attention.

The role of protein in energy regulation is presented in Chapter 1, with a number of potential targets in the energy balance equation identified, notably enhancement of satiety and energy intake, possibly through an effect on gastric emptying, gastrointestinal hormone release and glucose metabolism, and an increase in energy expenditure and alteration of substrate oxidation rates. The potential benefit of protein on metabolic risk factors is also presented.

## 6.2 Satiety and energy intake

The work presented in Chapter 3 described a classic preload design study which investigated short term satiety and energy intake after high-protein meals of different protein type and a control, taking into account the benefits and restrictions of this type of study performed in a metabolic suite setting. The study identified that when high-protein meals differing in protein type but designed to be as familiar as possible, are provided with the percentage of protein just above the recommended upper limit of daily intake, soy protein stimulates satiety and inhibits food intake, while nuts and legumes enhance meal satiation.

In the longer term study presented in Chapter 4 a high-protein diet over 3 days containing protein at the upper limit of the recommended range, increased meal satiation, enhanced fullness and reduced energy intake by 0.6 MJ/d. In contrast, a reduction in dietary protein to the lower limit of the recommended range did not alter appetite sensations or energy intake when compared to a diet of standard protein content, despite predictions from the protein leverage hypothesis, described in Chapter 4, that lower availability of protein would lead to a compensatory increase in carbohydrate and fat consumption to reach a specific protein intake target.

The effect of protein to increase satiety and reduce energy intake was investigated further to identify whether plausible mechanisms exist to account for such an effect and to predict whether consumption of high-protein diets in the long term may produce a sustained physiological suppression of energy intake. As presented in Chapter 3, a slower rate of gastric emptying seen after the soy protein diet may be a mechanism by which soy protein increases satiety and reduces energy intake. Gastrointestinal hormones slow gastric emptying, and while these hormones were not measured simultaneously with gastric emptying, measurements after high and low-protein meals in Chapter 4 demonstrated greater secretion of PP and the trend to greater GLP-1 secretion after a high-protein diet. Infusions of gastrointestinal hormones increase satiety and the ability of diets high in protein to alter their release may be one of the predominant mechanisms by which protein influences satiety and energy intake.

In Chapters 3 and 4 measurement of post-prandial glucose, which is known to stimulate hunger when concentrations increase and decrease rapidly, suggested that diets high in protein have an important role in regulating post-prandial glucose measurements particularly when the protein type is from plant source (for example, soy or nuts and legumes). The reduction in post-prandial glucose concentration coincided with an increase in satiety and a reduction in energy intake and provides further evidence that plausible physiological mechanisms exist to account for the reduction in EI with an increase in the proportion of dietary protein.

Whether the observed reduction in energy intake would persist in the long term is debatable and requires further clarification. The studies reported in this thesis were performed under highly controlled conditions, which demonstrate the physiological effects of the alteration in diet, but is an environment distinctly different to usual eating

habits. The reduction in daily energy intake of 0.6 MJ/d equating to a loss of body mass of 3.7 kg over six months is equivalent to some weight loss intervention trials (Samaha *et al.*, 2003). This degree of loss of body mass compares favourably to *ad libitum* low fat, high carbohydrate diets (Astrup *et al.*, 2000) and indeed is greater than in some studies that have manipulated the fat and carbohydrate content and source during *ad libitum* diets (Saris *et al.*). However, providing evidenced based dietary guidelines is limited by lack of long term effects, with initial loss in body mass, usually over six months, rarely persisting past one year (Sacks *et al.*, 2009).

A change in habitual eating patterns must therefore be acceptable and sustainable. The DiOGenes study is the only UK trial to focus specifically on weight maintenance (after a period of weight loss) with high or low protein diets in overweight adults. Fewer dropouts occurred during the high protein groups and together with a reduction in weight regain (Larsen *et al.*, in press) these data suggest that a moderate increase in protein may be acceptable long term and may be important in the prevention of weight gain. Additionally this study demonstrates that the satiating properties of protein exist in overweight subjects, suggesting that the results of the studies in this thesis performed in lean subjects may be extrapolated to the overweight or obese.

## 6.3 Energy expenditure and substrate oxidation

Chapter 5 describes a highly standardised study investigating energy expenditure during diets differing in their protein:energy ratio. Providing meals of 10%, 15% or 25% of energy from protein demonstrated that there was no difference in any parameter of energy expenditure, and clearly shows that any observed reduction in body mass during diets of moderately high-protein is not explained by alterations in energy expenditure.

However, the percentage of protein in the diet has a significant impact on substrate oxidation rates. This study confirmed that protein and carbohydrate oxidation is autoregulated, while that of fat, as the lowest macronutrient in the oxidation hierarchy, is dependent on the oxidation of the other two substrates. Fat oxidation is greater when protein intake is higher (and carbohydrate intake lower) than when the same fat intake occurs in a diet low in protein, a finding which could have positive long-term benefits on loss of body mass and in particular a reduction in fat storage. While reduction in carbohydrate has been recognised as a critical factor in regulating fat oxidation, especially during over- or under-feeding, it is worthwhile exploring further whether protein *per se* may alter fat oxidation, enabling higher-protein diets to reduce fat mass and preserve lean mass during weight loss.

## 6.4 Body mass and composition

Despite greater fat oxidation observed during the high-protein diet there was no consistent reduction in body mass or the measurements that reflect fat mass - waist circumference, air displacement plethysmography or DXA scanning - in the studies reported in Chapters 4 and 5. The treatment periods and time between measurements were short and it was therefore not unexpected to find that these parameters remained unaltered. Most of the previous work investigating changes in body composition with a high-protein diet has occurred over longer periods of time, usually during weight reduction and applied a higher percentage of energy from protein than was used in this study. Chapter 5 suggests a plausible mechanism for a dose dependent increase in fat oxidation with increasing protein consumption. Whether moderate increases in protein consumption over a longer time period would lead to a dose-dependent reduction in fat

mass or body mass is of interest, given the potential metabolic benefits of reducing fat mass by adopting a simple and acceptable dietary change.

## 6.5 Energy balance and appetite control mechanisms

These studies indicate that dietary protein regulates energy balance by altering energy intake but has no effect on energy expenditure. In contrast it appears that small changes in protein intake (and indeed in protein type) can alter energy intake and do so through a combination of changes in satiety and metabolic factors that influence appetite. The relative importance of the factors observed in these studies that reduce appetite – slowing of gastric emptying, increased secretion of anorexigenic gastrointestinal hormones, and reduced glycaemic excursions – is difficult to ascertain, not least because these factors are intrinsically intertwined. A slow rate of gastric emptying increases satiety and additionally may reduce the rate at which glucose appears in the blood after a meal. Gastrointestinal hormones (amongst other functions) stimulate anorexigenic centres in the brain to reduce energy intake, act locally to reduce the rate of gastric emptying, and, in the case of GLP-1, stimulate insulin secretion in the pancreas to reduce blood glucose levels.

## 6.6 Metabolic risk

The relationships between protein quantity and type and markers of metabolic risk were explored in the studies reported in Chapters 3 and 4. In Chapter 3, the action of protein type on glucose and insulin metabolism was clearly demonstrated. In particular, beneficial post-prandial effects of nuts and legumes and soy were observed on blood glucose. This finding provides a mechanism for the metabolic and cardiovascular benefits observed with

habitual diets high in nuts or soy, and suggests that dietary recommendations to alter protein intake should also involve guidance on optimal protein composition.

The relationship between dose-dependent changes in protein and glucose and insulin metabolism was explored further. In Chapters 4 and 5, the post-breakfast glucose and insulin curves did not differ between the diets, possibly due to the mixed protein source of the breakfast meal, particularly the use of dairy proteins which were found to have greater post prandial glucose concentrations in Chapter 3, and because the breakfast meal was lower than one third of the daily energy content. The results reported in Chapter 4 show clear dose-dependent differences in 24 h glucose profiles with changes in the protein composition of the diet. The importance of glucose concentrations in the development of the metabolic syndrome, as described in Chapter 1, imply that altering protein intake may be advantageous in the long-term modification of metabolic risk.

That relatively small change in dietary protein can produce clear and clinically significant changes in glucose concentrations, in lean healthy subjects, is important. The relationship between elevated glucose and metabolic risk factors appears to occur in a continuous manner, rather than a specific cutoff value existing which prevents the development of metabolic disease. The ability to reduce glucose concentrations, even within the normal range, is therefore valuable and may be the most clinically significant finding of these studies. Whether these improvements in metabolic risk factors, due to moderate changes in dietary protein, lead to reductions in morbidity and mortality from metabolic diseases needs to be explored further in larger, long-term trials.

## 6.7 Future directions

The search for appropriate strategies to reduce the ever-increasing burden of obesity has led to a considerable amount of research into dietary approaches that may prevent the development of overweight and obesity, assist in regimes to reduce body mass in those currently overweight, and prevent the development of weight-related metabolic disease. The challenge is to identify a dietary strategy that can be implemented easily and is sustainable.

An aspect that remains unclear from the work presented in this thesis is whether the beneficial effects observed from a moderate increase in protein is due primarily to the protein *per se* or because of a reciprocal reduction in carbohydrate. Fat was deliberately kept constant in these studies, leading to subjects consuming differing levels of carbohydrate, a factor which is known to affect post-prandial glucose excursions and fat oxidation. Simply reversing the diets to keep carbohydrate constant instead of fat would not solve this problem as increasing fat is associated with hyperphagia, changes in gastric emptying, and deterioration in lipid profiles. An approach would be to design a study based on low- or high- protein and include diets both high and low in fat and carbohydrate for each protein level. This larger study would require more subjects and ideally be of longer duration.

One way to clarify whether differing dietary protein leads to long-term changes in energy regulation is to compare changes in body mass in subjects whose protein intake can be objectively measured. Urinary nitrogen excretion reliably reflects protein intake and so could be used to identify whether changes in body mass are due to actual protein consumed (rather than a randomised intervention group which may not have complete adoption of the study diet). This information is available in the long-term intervention

study DiOGenes where an analysis of change in body mass relative to urine nitrogen excretion is planned.

Long-term interventions are important to determine whether any of the effects of altering dietary protein observed in a metabolic suite environment translates to similar results in free-living subjects. Greater longevity of the study can more easily clarify whether the intervention diets are acceptable, sustainable and produce positive outcomes. Such comparisons require large sample sizes necessitating multi-centre projects, carefully standardised study design and monitoring.

The work presented in this thesis suggests that small increases in dietary protein may be an appropriate approach to assist in maintaining energy regulation. Moderately high protein meals are straightforward to prepare with standard ingredients, are palatable and acceptable and could therefore be predicted to be able to be adopted in the long term. The question remains as to whether this is indeed feasible in the wider population and whether a widespread recommendation to increase protein would have any deleterious effects in the community. Experience has shown us that changing habitual diet is challenging. The general understanding of nutrition in the community is limited, and therefore advice to alter protein intake would require education about what constitutes dietary protein and how to assess the quantities that need to be consumed. Expecting individuals to 'count' protein grams would be untenable and a simpler message would need to be provided. In an experimental setting, advice to alter the proportion of protein in the diet has been successful, even when used across eight European countries (Moore *et al.*, 2010). The strategy used in this setting required subjects to work closely with a dietitian to understand protein exchanges, however it is feasible that a simpler version could be developed to provide relatively straightforward messages for the wider population.

Alongside an increase in education regarding a change in protein intake individuals may be assisted by the food industry providing high protein meals. Interestingly Marks and Spencer have recently introduced a high protein range, marketed to increase satiety for use as a weight loss strategy, with protein consumption recommended to be 30% of total energy. Slimfast have a similar approach for their rapid weight loss meal replacement plans with recommendations for high protein consumption. It is foreseeable that other food manufacturers will follow this approach and include high protein meals in their standard ranges for weight control, rather than just targeted for weight loss.

However, relying on manufactured products to alter eating habits provides two major problems. Firstly, education regarding ideal diet composition would not improve and people become more reliant on ready-made meals, further limiting the ability to cook and use fresh ingredients. There already exists a substantial problem with poor food preparation skills and complicating the dietary recommendations would undoubtedly worsen this. Secondly, ready-made meals are costly and are less accessible to low-income groups - the groups that are most vulnerable to developing obesity and metabolic diseases. Indeed it could be argued that adopting a high protein diet, whether relying on pre-prepared meals or fresh produce, may be too expensive for many people in the community. This is undoubtedly true if the source of protein is of animal origin, however non-animal sources of protein – beans, legumes, seeds, nuts, soy, etc – are generally affordable and, as was presented in this thesis, are associated with lower risk factors for metabolic disease. A simple recommendation to increase consumption of these non-animal sources of protein may have significant health benefits.

Further issues exist when considering widespread increases in protein consumption. It is likely that protein of animal origin would increase, leading to increased demand for

animal production and the cereal production to feed these animals, with consequences for scarce resources. The humane treatment of farmed animals has come under debate recently with current farming practices already causing concern. Increasing the demand for animal products would likely pose further ethical dilemmas for the farming industry. Additionally, there are significant concerns about deforestation and the environmental effects of the need for an increase in farmland. At a local level converting forested areas to farmland may seem economically beneficial, however on a large scale further destruction of forests would produce disastrous environmental consequences.

The problem of obesity is large and calls for a concerted response and comprehensive recommendations for appropriate dietary strategies. It is hoped that the work presented in this thesis may contribute to the development of such strategies.

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# Appendices

## Appendix 1: The effect of protein type on short-term appetite and energy intake

### I.1 Preload Meals

### I.2 Study questionnaires and forms

*I.2.1 Information Sheet*

*I.2.2 Consent Form*

*I.2.3 Telephone Screening Questionnaire*

*I.2.4 Approach letter to participants*

*I.2.5 Letter to participant's GP*

*I.2.6 Advertisement*

*I.2.7 Appetite Questionnaire*

*I.2.8 Palatability Questionnaire*

**Meat Control**

| | | | | | | | | Fatty acids | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Ingredients | Protein | Fat | Carbohydrate | Energy | Dietary Fibre | Saturated | Monounsaturated | Polyunsaturated | Trans. |
| | g | g | g | g | kJ | g | g | g | g | g |
| Beef lean raw | 28 | 6.3 | 1.2 | 0 | 152 | 0 | 0.5 | 0.5 | 0.1 | 28.0 |
| Egg yolk | 18 | 2.9 | 5.5 | 0 | 252 | 0 | 1.6 | 2.4 | 0.6 | 0 |
| Egg white | 32 | 2.9 | 0 | 0 | 49 | 0 | 0 | 0 | 0 | 0 |
| Tomatoes, canned | 250 | 2.5 | 0.3 | 7.5 | 173 | 1.8 | 0 | 0 | 0 | 0 |
| Spinach, raw | 90 | 2.5 | 0.7 | 1.4 | 93 | 1.9 | 0.1 | 0.1 | 0.5 | 0 |
| Carrots | 150 | 0.9 | 0.5 | 11.9 | 219 | 3.6 | 0.2 | 0 | 0.5 | 0 |
| Olive oil | 14.5 | 0 | 14.5 | 0 | 536 | 0 | 2.1 | 10.6 | 1.2 | 0 |
| Mixed herbs | | | | | | | | | | |
| Potato flakes | 65 | 4.6 | 0.3 | 54.3 | 1012 | 1.0 | 0.1 | 0 | 0 | 0 |
| Water to mix | 310 | | | | | | | | | |
| **Sum g** | | **22.5** | **22.9** | **75.1** | | **8.3** | **4.5** | **13.6** | **2.8** | **0** |
| **Sum kJ** | | **383** | **848** | **1276** | **2508** | | | | | |
| **% energy** | | **15.3** | **33.8** | **50.9** | **100** | | | | | |

## Meat high-protein

| | Ingredients | Protein | Fat | Carbohydrate | Energy | Dietary Fibre | Fatty acids | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Saturated | Monounsaturated | Polyunsaturated | Trans |
| | g | g | g | g | kJ | g | g | g | g | g |
| Beef lean, raw | 150 | 33.8 | 6.5 | 0 | 813 | 0 | 2.6 | 2.9 | 0.3 | 0.2 |
| Egg yolk | 18 | 2.9 | 5.5 | 0 | 252 | 0 | 1.6 | 2.4 | 0.6 | 0 |
| Egg white | 32 | 2.9 | 0 | 0 | 49 | 0 | 0 | 0 | 0 | 0 |
| Tomatoes, canned | 250 | 2.5 | 0.3 | 7.5 | 173 | 1.8 | 0 | 0 | 0 | 0 |
| Spinach raw | 120 | 3.4 | 1.0 | 1.9 | 124 | 2.5 | 0.1 | 0.1 | 0.6 | 0 |
| Olive oil | 10 | 0 | 10.0 | 0 | 370 | 0 | 1.4 | 7.3 | 0.8 | 0 |
| Mixed Herbs | | | | | | | | | | |
| Potato flakes | 46 | 3.2 | 0.2 | 38.4 | 716 | 0.7 | 0.1 | 0 | 0 | 0 |
| Water to mix | 220 | | | | | | | | | |
| **Sum g** | | **48.6** | **23.4** | **47.8** | | **5.0** | **5.8** | **12.6** | **2.3** | **0.2** |
| **Sum kJ** | | **824** | **865** | **813** | **2504** | | | | | |
| **% energy** | | **33.0** | **34.5** | **32.5** | **100** | | | | | |

## Dairy

| | Ingredients | Protein | Fat | Carbohydrate | Energy | Dietary Fibre | Fatty acids | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Saturated | Monounsaturated | Polyunsaturated | Trans |
| | g | g | g | g | kJ | g | g | g | g | g |
| Cottage cheese, reduced fat | 200 | 26.6 | 3.0 | 6.6 | 668 | 0 | 2.0 | 0.8 | 0 | 0 |
| Cheddar, half fat | 28 | 9.2 | 4.4 | 0 | 320 | 0 | 2.8 | 1.3 | 0.1 | 0 |
| Egg yolk | 18 | 2.9 | 5.5 | 0 | 252 | 0 | 1.6 | 2.4 | 0.6 | 0 |
| Egg white | 32 | 2.9 | 0 | 0 | 49 | 0 | 0 | 0 | 0 | 0 |
| Tomatoes, canned | 100 | 1.0 | 0.1 | 3.0 | 69 | 0.7 | 0 | 0 | 0 | 0 |
| Spinach raw | 110 | 3.1 | 0.9 | 1.8 | 113 | 2.3 | 0.1 | 0.1 | 0.6 | 0 |
| Olive oil | 9 | 0 | 9.0 | 0 | 333 | 0 | 1.3 | 6.6 | 0.7 | 0 |
| Mixed Herbs | | | | | | | | | | |
| Potato flakes | 44 | 3.1 | 0.2 | 36.7 | 685 | 0.7 | 0.1 | 0 | 0 | 0 |
| Water to mix | 220 | | | | | | | | | |
| **Sum g** | | **48.7** | **23.1** | **48.1** | | **3.7** | **7.8** | **11.1** | **2.0** | **0** |
| **Sum kJ** | | **828** | **855** | **818** | **2500** | | | | | |
| **% energy** | | **33.1** | **34.2** | **32.7** | **100** | | | | | |

## Nuts and legumes

| | | | | | | | Fatty acids | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ingredients | Protein | Fat | Carbohydrate | Energy | Dietary Fibre | Saturated | Monounsaturated | Polyunsaturated | Trans |
| | g | g | g | g | kJ | g | g | g | g | g |
| Almonds | 29 | 6.0 | 15.9 | 2.0 | 722 | 2.1 | 1.3 | 9.8 | 4.0 | 0 |
| Lentils, brown, raw | 75 | 15.3 | 1.2 | 30.7 | 796 | 5.6 | 0.1 | 0.2 | 0.5 | 0 |
| Egg yolk | 18 | 2.9 | 5.5 | 0 | 252 | 0 | 1.6 | 2.4 | 0.6 | 0 |
| Egg white | 192 | 17.3 | 0 | 0 | 294 | 0 | 0 | 0 | 0 | 0 |
| Tomatoes, canned | 120 | 3.0 | 0.3 | 9.0 | 207 | 2.1 | 0 | 0 | 0 | 0 |
| Spinach raw | 80 | 2.8 | 0.8 | 1.6 | 103 | 2.1 | 0.1 | 0.1 | 0.5 | 0 |
| Mixed herbs | | | | | | | | | | |
| Potato flakes | 6 | 0.5 | 0.1 | 5.9 | 109 | 0.1 | 0 | 0 | 0 | 0 |
| Water to mix | 28.5 | | | | | | | | | |
| **Sum g** | | **47.8** | **23.8** | **49.2** | | **12.0** | **3.1** | **12.5** | **5.7** | **0** |
| **Sum kJ** | | **812** | **880** | **836** | **2528** | | | | | |
| **% energy** | | **32.1** | **34.8** | **33.1** | **100** | | | | | |

## Soy

| | Ingredients | Protein | Fat | Carbohydrate | Energy | Dietary Fibre | Fatty acids | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Saturated | Monounsaturated | Polyunsaturated | Trans |
| | g | g | g | g | kJ | g | g | g | g | g |
| Soy mince dry | 49 | 18.5 | 0.4 | 16.3 | 684 | 1.5 | 0 | 0 | 0 | 0 |
| Tofu blue dragon | 230 | 15.9 | 6.2 | 5.5 | 593 | 0.2 | 0.9 | 3.4 | 1.2 | 0 |
| Egg yolk | 18 | 2.9 | 5.5 | 0 | 252 | 0 | 1.6 | 2.4 | 0.6 | 0 |
| Egg white | 32 | 2.9 | 0 | 0 | 49 | 0 | 0 | 0 | 0 | 0 |
| Tomatoes canned | 180 | 1.8 | 0.2 | 5.4 | 124 | 1.3 | 0 | 0 | 0 | 0 |
| Spinach raw | 180 | 5.0 | 1.4 | 2.9 | 185 | 3.8 | 0.2 | 0.2 | 0.9 | 0 |
| Olive oil | 9 | 0 | 9.0 | 0 | 333 | 0 | 1.3 | 6.6 | 0.7 | 0 |
| Mixed Herbs | | | | | | | | | | |
| Potato flakes | 22.5 | 1.6 | 0.2 | 18.9 | 350 | 0.4 | 0 | 0 | 0 | 0 |
| Water to mix | 110 | | | | | | | | | |
| **Sum g** | | **48.6** | **22.9** | **49.0** | | **7.1** | **4.0** | **12.6** | **3.5** | **0** |
| **Sum kJ** | | **826** | **848** | **833** | **2506** | | | | | |
| **% energy** | | **32.9** | **33.8** | **33.2** | **100** | | | | | |

You are invited to take part in a research study. Before you decide whether or not you wish to take part it is important that you understand what the research will involve. Please read this information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Please take as much time as you would like.

**What is the purpose of this study?**
Eating is a complex interaction between many different factors. We know in general what happens when we eat, but little is known about how our body responds to different types of food. By giving you five different meals, and looking at metabolic factors and hormones in your blood, and working out how fast your stomach empties, we will look more closely at what happens when we eat certain types of food.

**Why have I been chosen?**
You have been contacted because you have expressed an interest in our research. We need healthy men or women between the ages of 18 to 70 years who are of normal weight, are non smokers, and are not pregnant. We will be recruiting up to 30 volunteers.

**Do I have to take part?**
It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. However, you will still be free to withdraw at any time and without giving a reason. You will not be able to take part if there are any foods that you cannot eat (eg. nuts, meat, dairy products).

**What will happen to me if I take part?**
You will be asked to attend MRC HNR five times with at least a week between visits. At the first visit you will complete a questionnaire about your eating habits and have your weight, height and blood pressure taken. Your weight will be recorded on every subsequent visit. You will have a small plastic tube (cannula) inserted into your vein with a needle. The needle will be removed and we will then be able to take blood samples from the cannula during the day without requiring any other needles. You will be given a meal to eat at 9am which you must finish. The meal will contain a marker – 13C-Octanoic acid. Octanoic acid is a naturally occurring fatty acid and 13C is a type of carbon present in the air we breathe. It is safe and not radioactive. When the meal leaves your stomach the 13C will break away and appear in your breath. From this we can tell how long the food has been in your stomach.
We will take blood samples from the cannula on 9 occasions (a total of 120mls of blood which is similar to about half a cup) from when you first arrive until midday. We will ask you to breathe through a straw into small plastic tubes, and to complete simple questionnaires during the morning.
At 12pm you will be given another meal and further questionnaires to complete.
Each visit will last about six hours.

**What do I have to do?**
We will ask you to continue with your usual exercise and diet habits, and not start or stop taking any dietary supplements during the study. It is especially important that the day before the investigation days you have a normal eating pattern, don't drink any alcohol, and don't do any vigorous exercise. It is important that you do not eat from 8pm the night before your visit. On the morning of your tests you can have one glass of water and you should avoid exercise.

**What are the possible benefits?**
You will have your blood pressure, weight, glucose and insulin levels measured, which will screen for your risk of high blood pressure and diabetes. There will be no other direct benefit to you. However, knowledge gained from this study will help our research into how our body responds to different types of food.

**What are the possible risks and disadvantages?**
There is a risk of bruising and brief discomfort when a cannula is inserted into your vein. There is a risk of an allergic reaction to the food if you have a food allergy, so we will ask you specifically about this before the study.
If a new diagnosis of diabetes or high blood pressure is made, this could affect your future insurance status (e.g. for life insurance or private medical insurance).

**What will happen if anything goes wrong?**
If something goes wrong during an investigation day any procedures will be stopped and you will be seen by the MRC HNR clinician. Your involvement in the rest of the study may be stopped.
If you have any other problems, illnesses or concerns during the study you should discuss these with the investigator.
Any complaints you have about this study will be fully investigated. For research carried out at MRC HNR participants would be in the same position as if public liability insurance had been taken out. If you wish to make a complaint you can contact our Unit Manager.

**Will my taking part in this study be kept confidential?**
Any information that is collected about you during the study will be kept strictly confidential. With your permission your GP will be notified that you are participating in this study.

**What will happen to the study results and blood samples?**
We will inform you and your GP of your blood pressure, weight, glucose and insulin results. As the blood samples will not be processed until the end of the study, your results will not be available for about six months. With your permission blood samples will be kept for 10 years for use in future nutrition studies. The overall results may be presented at scientific meetings or published in a scientific journal. You will not be identified in any of these presentations or publications. A summary of the study results will be available on MRC HNR website and we will be happy to discuss the results with you at the end of the study.

**Will I be reimbursed for my time?**
In recognition of your time commitment, you will be paid an honorarium of £30.00 per visit. Reasonable travel expenses will also be paid.

**Who is organising and funding the study?**
This study is being organised by the Nutrition and Health and Stable Isotope group at MRC-HNR. The Medical Research Council is funding the research.

**Who has reviewed the study?**
This study has been reviewed by the Scientific Co-ordination Committee of MRC HNR and by Peterborough and Fenland Local Research Ethics Committee.

**Further information or questions?**
Please contact Rosemary Hall at MRC HNR on 01223 426356 or Email rosemary.hall@mrc-hnr.cam.ac.uk

**And finally...**
Thank you for taking the time to read this and your interest in the study. If you decide to take part in the study, you will be given a copy of this information sheet and a signed consent form.

MRC | Human Nutrition Research

# Metabolic Response to different Meal Types

## Information Sheet for Study Participants

Chief Investigator: Dr Rosemary Hall

MRC Human Nutrition Research
Elsie Widdowson Laboratory
Fulbourn Road
Cambridge
CB1 9NL

Telephone: 01223 437611
Fax: 01223 437515
research@mrc-hnr.cam.ac.uk
http://www.mrc-hnr.cam.ac.uk

Version 03, 25 August 2005

# Metabolic Response to different Meal Types

## CONSENT FORM

LREC Reference Number: 05/Q0106/37
Name of Lead Investigator: Dr Rosemary Hall

**Please initial box**

1. I confirm that I have read and understand the information sheet dated August 2005 (version 03) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my legal rights being affected. ☐

3. I consent to my general practitioner being notified of my participation in this research. ☐

4. I consent to my general practitioner being notified of my study results and any abnormalities found during this research. ☐

5. I understand that samples taken as part of the protocol of this study may be stored for up to 10 years and used in further research studies. Samples will only be used in studies that have been approved by the appropriate Ethics Committee. ☐

6. I understand that samples taken as part of this study may be analysed in another laboratory outside of MRC Human Nutrition Research. All samples will be made anonymous, and no personal information will be sent to another laboratory. ☐

7. I understand that I cannot participate in this research if I am pregnant. I am not pregnant and will inform the research team if I become pregnant. ☐

8. I agree to take part in the above study. ☐

| Name of Volunteer *(Please print)* | Date | Signature |
|---|---|---|
| Name of Research Team Member *(Please print)* | Date | Signature |
| Name of Researcher *(Please print)* | Date | Signature |

***3 copies required:*** top copy for researcher; one copy for volunteer; one copy to be kept with research subject's notes.

# Metabolic Response to different Meal Types

## Telephone Screening Questionnaire

Name .....

Address .....

.....

Age ..... Date of Birth .....

Phone Number: Day ..... Evening .....

Best time to phone .....

General Practitioner .....

Of (medical practice).....

GP telephone number.....

How did you hear about the study? (Advertisement/Database/etc).....
Are you currently in any other research studies, or have you been in any in the last year?
.....

**Health**

Do you have or have you had any of the following conditions?

| | | YES | NO |
|---|---|---|---|
| (i) | Diabetes | ☐ | ☐ |
| (ii) | Stomach or bowel problems | ☐ | ☐ |
| (iii) | Asthma/Eczema/Hayfever | ☐ | ☐ |
| (iv) | High Cholesterol | ☐ | ☐ |
| (v) | Chronic medical conditions | ☐ | ☐ |
| (vi) | Anaemia | ☐ | ☐ |
| (vii) | Cancer | ☐ | ☐ |
| (viii) | Food/Drug allergies | ☐ | ☐ |
| (ix) | Eating Disorder | ☐ | ☐ |
| (viii) | Are you Pregnant (or planning) or Breastfeeding | ☐ | ☐ |
| (ix) | Are you premenopausal | ☐ | ☐ |

Please list any others .....
.....

Please list any medications (pills, tablets, contraceptives, inhalers etc.) that you are taking, either prescribed by your doctor or purchased over the counter.
.....
.....

Current Weight ..... Height ..... Estimated BMI .....

| | YES | NO |
|---|---|---|
| Do you smoke?<br>If yes, how many cigarettes per day? ..... | ☐ | ☐ |
| Do you drink alcohol?<br>If yes, how many units of alcohol would you consume in an average week?<br>(1 unit = 1 measure of spirits / 1 small glass of wine / 1 half pint of beer)<br>..... | ☐ | ☐ |

**Availability and Transport**

| | YES | NO |
|---|---|---|
| Would you have any difficulty in: | | |
| 1. Attending MRC-HNR on five occasions? | ☐ | ☐ |
| 2. Eating all types of food? | ☐ | ☐ |
| 3. Having a blood sample taken? | ☐ | ☐ |
| 4. Fasting overnight? | ☐ | ☐ |
| 5. Breathing into a tube through a straw? | ☐ | ☐ |
| Would you have any anticipated difficulties with your availability or transport arrangements to attend each of these sessions? | ☐ | ☐ |
| Do you have any preferred days to attend the unit?<br>If yes, please give days.<br>..... | ☐ | ☐ |

Name of Research Team Member *(Please print)* _____ Date _____ Signature _____

Eligible for this study YES NO

If YES:
- Send Information Sheet..... YES NO
- Discussed supplement use YES NO
- Discussed maintenance of lifestyle factors YES NO
- If pre-menopausal discussed menstrual phase YES NO

.....

Appointments
- Visit 1.....
- Visit 2.....
- Visit 3.....
- Visit 4.....
- Visit 5.....

If NO: Would you like information about other studies at MRC-HNR or consider placing your name on our Volunteer Database? YES NO

Questionnaire Sent..... YES NO

**Address**

**Date**

Dear

## Re: Metabolic Response to different Meal Types

We are currently recruiting volunteers for a nutrition study at MRC Human Nutrition Research. We have looked at the information that we have on our volunteer database and found that you may be able to be involved.

This study will investigate the way our body responds to different types of food.

We are looking for a total of 30 healthy volunteers for this study. Each volunteer would be required to attend MRC Human Nutrition Research for six hours on five different occasions. You would be given different meals and we will measure what happens to hormones and metabolic factors in your blood after the meals.

In recognition of your time commitment, you will receive an honorarium of £10 for each visit. Reasonable travel expenses will also be reimbursed.

Please read the enclosed volunteer information sheet for more details, and if you are interested in taking part, or if you have any questions, please contact me on 01223 426356.

Kind regards

Dr Rosemary Hall
Clinical Scientist

Dr. .....
*GP Address*

*Date*

Dear Dr .....,

**Re: Metabolic Response to different Meal Types**

Your patient *Name, DOB & Address of Volunteer,* has volunteered to take part in a research study at MRC Human Nutrition Research (HNR). *He/She* has given us permission to contact you.

The study is designed to investigate metabolic response, satiety hormones levels, and gastric emptying effects from eating various meal types. (For more information see the attached volunteer information sheet). Participants will need to attend MRC Human Nutrition Research on five occasions and will be provided with different meals on each occasion. A cannula will be inserted for blood sampling and participants will be required to perform frequent breath tests throughout the test days.

Your patient has been asked to provide us with their medical history. Participants will be excluded if they have any acute or chronic disease, are pregnant, breastfeeding or planning pregnancy, or are taking any medication other than an oral contraceptive pill.

We will inform you if any abnormal results are obtained which may be of clinical significance.

**If you feel that there is a medical reason which should prevent their participation in this study or if you need further details please contact me on 01223 - 426356.**

Yours sincerely

Dr Rosemary Hall
Clinical Scientist

MRC | Human Nutrition Research

# Are You Interested in Nutrition Research?

We are running a study to investigate how our body responds to different meals

---

**Are you:**

**Aged 18-70 years**

**Healthy, non smoker, and a normal weight**

**Willing to eat different types of food**

- including meat and nuts

**Able to attend MRC HNR five times**

**Willing to give blood samples**

---

**If you would like more information please contact Rosemary Hall on (01223) 437611**

rosemary.hall@mrc-hnr.cam.ac.uk

You will be reimbursed for participation in the study and for reasonable travel expenses

Version 02, 25 April 2005

**Appetite Questionnaire**

Date ☐☐/☐☐/☐☐

Participant ID ☐☐☐☐☐

Visit No: _____ Timepoint: _____ min

- Please answer the following questions by placing a vertical mark through the line.
- Regard the ends of each line as indicating the most extreme sensation you have ever felt.

1. How **hungry** are you?

   Not at all hungry _____ Very hungry

2. How **full** do you feel?

   Not at all full _____ Very full

3. How strong is your **desire to eat**?

   Not at all strong _____ Very strong

4. How **much food** do you think you could eat?

   None at all _____ A large amount

5. How **contented** are you?

   Not at all contented _____ Very contented

6. How **irritable** are you?

   Not at all irritable _____ Very Irritable

7. How **depressed** are you?

   Not at all depressed _____ Very depressed

8. How **mentally alert** are you?

   Not at all Alert _____ Very Alert

**Meal Questionnaire**

Date ☐☐/☐☐/☐☐

Participant ID ☐☐☐☐☐

Visit No: _____ Timepoint: _____ min

Please answer the following questions by placing a vertical mark through the line.
Regard the ends of each line as indicating the most extreme sensation you have ever felt.

1. How **sweet** did you find the meal?
   Not at all sweet _____ Extremely sweet

2. How **savoury** did you find the meal?
   Not at all savoury _____ Extremely savoury

3. How **tasty** did you find the meal?
   Not at all tasty _____ Extremely tasty

4. How **pleasant** did you find the meal?
   Not at all pleasant _____ Extremely pleasant

5. How **filling** did you find the meal?
   Not at all Filling _____ Extremely filling

6. How **satisfying** did you find the meal?
   Not at all satisfying _____ Extremely satisfying

7. How much more of **this** food do you think you could eat?
   A small amount _____ A large amount

8. How **enjoyable** did you find the meal?
   Not at all tasty _____ Extremely tasty

# Appendix II: Study 2 Measuring the effect of manipulating the P:E ratio on energy metabolism.

## II.1 Meals

## II.2 Study questionnaires and forms

### *II.2.1 Information Sheet*

### *II.2.2 Consent Form*

### *II.2.3 Telephone screening questionnaire*

### *II.2.4 Approach Letter to Participants*

### *II.2.5 Letter to participant's GP*

### *II.2.6 Advertisement*

### *II.2.7 Three Factor Eating Questionnaire*

### *II.2.8 Eating Attitude Test*

### *II.2.9 International Physical Activity Questionnaire*

### *II.2.10 Instructions for wearing the CGMS*

| **Meal Outline** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Day 0 Sun** | **Day 1 Mon** | **Day 2 Tue** | **Day 3 Wed** | **Day 4 Thur Cal** | **Day 5 Fri** | |
| | | ***ad lib.*** | ***ad lib.*** | ***ad lib.*** | **fixed-energy** | | **Energy offered during *ad lib.* days (kJ)** |
| **Breakfast** | - | Cereal | Porridge | Cereal | Porridge | Usual breakfast CRF | 3500 |
| **Daytime snack** | - | yoghurt muffins | tuna sandwiches | peanut butter sandwiches | | | 2500 |
| | | cheese scones | | | | | 2500 |
| **Lunch** | - | Macaroni cheese | Veg and lentil curry | Macaroni cheese | Veg and lentil curry | | 5000 |
| | | Raspberry yoghurt | Strawberry yoghurt | Raspberry yoghurt | Strawberry yoghurt | | 2500 |
| **Dinner** | standard dinner | Mushroom chicken and rice | Bolognaise pasta | Mushroom chicken and rice | Bolognaise pasta | | 5000 |
| | | Bread and butter pudding | Rice pudding | Bread and butter pudding | Rice pudding | | 2500 |
| **Evening Snack** | | muffins scones | muffins sandwiches | sandwiches scones | | | |
| | | yoghurt | yoghurt | yoghurt | | | 2500 |
| **Total** | | | | | | | **26000** |

| Sunday standard dinner | |
|---|---|
| | **Energy provided kJ** |
| Tescos Steam Chicken in mild coconut curry sauce 450 g | 1820 |
| Tescos Baby Leaf Salad with purple basil 85 g | 31 |
| Pudz Sticky Toffee Pudding 210 g | 3303 |
| | **5154** |
| **Evening Snack (optional)** | |
| Duchy Originals lemon biscuits | 1293 |
| | **6447** |

## Porridge

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| **porridge, Jordan's porridge oats** | 130 | 15.2 | 12.1 | 75.9 | 1996 | 11.7 |
| sugar, demerara | 19 | 0.1 | 0.0 | 19.9 | 319 | 0.0 |
| dried skimmed milk | 13 | 4.7 | 0.1 | 6.9 | 192 | 0.0 |
| raisins | 32 | 0.4 | 0.2 | 20.5 | 360 | 2.2 |
| butter | 20 | 0.1 | 16.4 | 0.1 | 612 | 0.0 |
| water | | | | | | |
| **Sum g** | | **20.5** | **28.8** | **123.3** | | **13.9** |
| **Sum kJ** | | **348** | **1064** | **2096** | **3508** | |
| **% energy** | | **9.9** | **30.3** | **59.7** | **100** | |
| **Meal B** | | | | | | |
| **porridge, jordans porridge oats** | 130 | 15.2 | 12.1 | 75.9 | 1996 | 11.7 |
| sugar, demerara | 20 | 0.1 | 0.0 | 20.9 | 336 | 0.0 |
| dried skimmed milk | 28 | 10.1 | 0.2 | 14.8 | 415 | 0.0 |
| Almonds | 25 | 5.3 | 14.0 | 1.7 | 634 | 1.9 |
| cream, single | 10 | 0.3 | 1.9 | 0.2 | 80 | |
| water | | | | | | |
| **Sum g** | | **31.0** | **28.1** | **113.6** | | **13.6** |
| **Sum kJ** | | **527** | **1040** | **1931** | **3499** | |
| **% energy** | | **15.1** | **29.7** | **55.2** | **100** | |
| **Meal C** | | | | | | |
| **porridge, jordans porridge oats** | 65 | 7.6 | 6.1 | 38.0 | 998 | 5.9 |
| sugar, demerara | 5 | 0.0 | 0.0 | 5.2 | 84 | 0.0 |
| milk, skimmed, | 550 | 18.7 | 1.1 | 24.2 | 748 | 0.0 |
| dried skimmed milk | 40 | 14.4 | 0.2 | 21.2 | 593 | 0.0 |
| Almonds | 37 | 7.8 | 20.7 | 2.6 | 938 | 2.7 |
| wheat bran | 11 | 1.6 | 0.6 | 3.0 | 96 | 4.0 |
| **Sum g** | | **50.1** | **28.6** | **94.1** | | **12.59** |
| **Sum kJ** | | **852** | **1060** | **1599** | **3510** | |
| **% energy** | | **24.3** | **30.2** | **45.5** | **100** | |

## Cereal

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| **Kellogg's All-bran bran flakes** | 135 | 13.5 | 2.7 | 90.5 | 1867 | 20.3 |
| Almonds | 15.5 | 3.3 | 8.6 | 1.1 | 393 | 1.1 |
| raisins | 45 | 0.5 | 0.2 | 28.8 | 507 | 3.1 |
| cream, single | 80 | 2.6 | 15.3 | 1.8 | 638 | |
| whole milk | 30 | 1.0 | 1.1 | 1.4 | 82. | |
| water to make 250 ml fluid | | | | | | |
| **Sum g** | | **20.9** | **28.0** | **123.5** | | **24.5** |
| **Sum kJ** | | **355** | **1037** | **2099** | **3491** | |
| **% energy** | | **10.2** | **29.7** | **60.1** | **100** | |
| **Meal B** | | | | | | |
| **kellogs all bran bran flakes** | 100 | 10 | 2 | 67 | 1383 | 15 |
| **kellogs all bran original** | 12 | 1.7 | 0.4 | 5.8 | 142.1 | 3.2 |
| milk, skimmed, | 100 | 3.4 | 0.2 | 4.4 | 136 | 0 |
| dried skimmed milk | 15 | 5.4 | 0.1 | 8.0 | 222 | 0 |
| Almonds | 46 | 9.7 | 25.7 | 3.2 | 1166 | 3.4 |
| raisins | 40 | 0.44 | 0.2 | 25.64 | 450.8 | 2.7 |
| **Sum g** | | **30.6** | **28.6** | **113.9** | | **24.4** |
| **Sum kJ** | | **521** | **1057** | **1936** | **3515** | |
| **% energy** | | **14.8** | **30.1** | **55.1** | **100** | |
| **Meal C** | | | | | | |
| **kellogs all bran bran flakes** | 35 | 3.5 | 0.7 | 23.5 | 484 | 5.3 |
| **kellogs all bran original** | 42 | 5.9 | 1.5 | 20.2 | 497 | 11.3 |
| milk, skimmed, | 200 | 6.8 | 0.4 | 8.8 | 272 | 0 |
| dried skimmed milk | 65 | 23.5 | 0.4 | 34.4 | 963 | 0 |
| Almonds | 45 | 9.5 | 25.1 | 3.1 | 1140 | 3.3 |
| wheat bran | 10 | 1.4 | 0.6 | 2.7 | 87 | 3.6 |
| water to make 250 ml fluid | | | | | | |
| **Sum g** | | **50.6** | **28.6** | **92.6** | | **23.56** |
| **Sum kJ** | | **859** | **1059** | **1574** | **3492** | |
| **% energy** | | **24.6** | **30.3** | **45.1** | **100** | |

## Yoghurt Muffins

| | Ingredients (g) | Ingredients (number) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|---|
| **Meal A** | | | | | | | |
| egg whole | 25 | 0.5 | 3.1 | 2.7 | 0.0 | 157 | 0.0 |
| raspberry Yeo Valley yoghurt | 50 | | 2.1 | 2.0 | 6.2 | 212.5 | 0.0 |
| flour white | 75 | | 7.1 | 1.0 | 58.3 | 1088 | 2.3 |
| baking powder | 2.5 | | 0.1 | 0.0 | 1.0 | 17.3 | 0.0 |
| butter | 18 | | 0.1 | 14.4 | 0.1 | 535 | 0.0 |
| sugar, white | 19 | | 0.1 | 0.0 | 19.9 | 319 | 0.0 |
| milk, skimmed, | 61 | | 2.1 | 0.1 | 2.7 | 83 | 0.0 |
| **Sum g** | | | **14.7** | **20.1** | **88.0** | | **2.3** |
| **Sum kJ** | | | **250** | **745** | **1496** | **2491** | |
| **% energy** | | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | | |
| egg white | 64 | 2 | 5.8 | 0.0 | 0.0 | 97.9 | 0.0 |
| raspberry Yeo Valley yoghurt | 50 | | 2.1 | 2.0 | 6.2 | 212.5 | 0.0 |
| almonds | 13 | | 2.6 | 7.0 | 0.9 | 316.8 | 0.9 |
| flour white | 55 | | 5.2 | 0.7 | 42.7 | 798 | 1.7 |
| baking powder | 2.5 | | 0.1 | 0.0 | 1.0 | 17 | 0.0 |
| butter | 13 | | 0.1 | 10.7 | 0.1 | 398 | 0.0 |
| sugar, white | 20 | | 0.1 | 0.0 | 20.9 | 336 | 0.0 |
| milk, skimmed, | 50 | | 1.7 | 0.1 | 2.2 | 68 | 0.0 |
| dried skimmed milk | 13 | | 4.5 | 0.1 | 6.6 | 185 | 0.0 |
| **Sum g** | | | **22.2** | **20.5** | **80.5** | | **2.6** |
| **Sum kJ** | | | **377.2** | **758.5** | **1368.2** | **2504** | |
| **% energy** | | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | | |
| egg white | 128 | 4 | 11.5 | 0.0 | 0.0 | 196 | 0.0 |
| raspberry Yeo Valley yoghurt | 25 | | 1.1 | 1.0 | 3.1 | 106 | 0.0 |
| natural Yeo Valley yoghurt | 50 | | 2.3 | 2.1 | 3.3 | 172 | 0.0 |
| almonds | 30 | | 6.3 | 16.7 | 2.1 | 760 | 2.2 |
| flour white | 52 | | 4.9 | 0.7 | 40.4 | 754 | 1.6 |
| baking powder | 2.5 | | 0.1 | 0.0 | 1.0 | 17.3 | 0.0 |
| milk, skimmed, 206 | 50 | | 1.7 | 0.1 | 2.2 | 68.0 | 0.0 |
| dried skimmed milk 229 | 25 | | 9.0 | 0.2 | 13.2 | 371 | 0.0 |
| **Sum g** | | | **36.9** | **20.7** | **65.2** | | **3.8** |
| **Sum kJ** | | | **627** | **767** | **1109** | **2503** | |
| **% energy** | | | **25** | **31** | **44** | **100** | |

## Scones

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| cheddar full fat | 20 | 5.1 | 7.0 | 0.0 | 345 | 0.0 |
| flour white | 214 | 20.1 | 2.8 | 166.3 | 3103 | 6.6 |
| baking powder | 15 | 0.8 | 0.0 | 5.7 | 104 | 0.0 |
| butter | 37 | 0.2 | 30.4 | 0.2 | 1132 | 0.0 |
| milk, skimmed, | 100 | 3.4 | 0.2 | 4.4 | 136 | 0.0 |
| **Sum g** | | **29.6** | **40.4** | **177** | | **6.6** |
| **Sum kJ** | | **503** | **1494** | **3002** | **4999** | |
| **% energy** | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | |
| Cheddar half fat | 64 | 20.9 | 10.1 | 0.0 | 730 | 0.0 |
| flour white | 193 | 18.1 | 2.5 | 150.0 | 2799 | 6.0 |
| baking powder | 15 | 0.8 | 0.0 | 5.7 | 104 | 0.0 |
| butter 351 | 34 | 0.2 | 27.5 | 0.2 | 1025 | 0.0 |
| milk, skimmed, 206 | 130 | 4.4 | 0.3 | 5.7 | 177 | 0.0 |
| **Sum g** | | **44.5** | **40.4** | **161.5** | | **6.0** |
| **Sum kJ** | | **756** | **1495** | **2746** | **4998** | |
| **% energy** | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | |
| Cheddar half fat | 163 | 53.3 | 25.8 | 0.0 | 1860 | 0.0 |
| flour white | 154 | 14.5 | 2.0 | 120.0 | 2233 | 4.8 |
| baking powder | 15 | 0.8 | 0.0 | 5.7 | 104 | 0.0 |
| wheat bran | 5 | 0.7 | 0.3 | 1.3 | 44 | 1.8 |
| butter 351 | 15 | 0.1 | 12.3 | 0.1 | 459 | 0.0 |
| milk, skimmed, 206 | 130 | 4.4 | 0.3 | 5.7 | 177 | 0.0 |
| **Sum g** | | **73.8** | **40.6** | **132.5** | | **6.6** |
| **Sum kJ** | | **1254** | **1503** | **2252** | **5009** | |
| **% energy** | | **25** | **30** | **45** | **100** | |

## Tuna sandwiches

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| white bread | 191 | 15.1 | 3.1 | 88.1 | 1778.2 | 3.6 |
| butter | 21 | 0.1 | 17.3 | 0.1 | 642.4 | 0.0 |
| tuna | 10 | 2.5 | 0.1 | 0.0 | 44.4 | 0.0 |
| **Sum g** | | **17.7** | **20.4** | **88.2** | | **3.6** |
| **Sum kJ** | | **301** | **754** | **1499** | **2465** | |
| **% energy** | | **12** | **30** | **59** | **100** | |
| **Meal B** | | | | | | |
| white bread | 175 | 13.8 | 2.8 | 80.7 | 1629.3 | 3.3 |
| butter | 21 | 0.1 | 17.3 | 0.1 | 642.4 | 0.0 |
| tuna | 34 | 8.5 | 0.2 | 0.0 | 151.0 | 0.0 |
| **Sum g** | | **22.5** | **20.2** | **80.8** | | **3.3** |
| **Sum kJ** | | **382** | **749** | **1374** | **2504** | |
| **% energy** | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | |
| warburtons wholemeal | 167 | 17.0 | 5.9 | 66.1 | 1629.9 | 10.5 |
| butter 351 | 17 | 0.1 | 14.0 | 0.1 | 520.0 | 0.0 |
| tuna | 79 | 19.8 | 0.4 | 0.0 | 350.8 | 0.0 |
| **Sum g** | | **36.9** | **20.2** | **66.2** | | **10.5** |
| **Sum kJ** | | **627** | **748** | **1126** | **2501** | |
| **% energy** | | **25** | **30** | **45** | **100** | |

## Peanut Butter Sandwiches

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| white bread | 180 | 14.2 | 2.9 | 83.0 | 1676 | 3.4 |
| butter | 15 | 0.1 | 12.3 | 0.1 | 459 | 0.0 |
| peanut butter tescos crunchy | 10 | 2.4 | 5.1 | 1.5 | 255 | 0.6 |
| **Sum g** | | **16.7** | **20.3** | **84.5** | | **4.1** |
| **Sum kJ** | | **283** | **752** | **1437** | **2472** | |
| **% energy** | | **11** | **30** | **58** | **100** | |
| **Meal B** | | | | | | |
| wholemeal bread | 1.85 | 17.4 | 4.6 | 77.7 | 1706 | 9.3 |
| butter | 5 | 0.0 | 4.1 | 0.0 | 153 | 0.0 |
| peanut butter tescos | 22 | 5.2 | 11.3 | 3.2 | 560 | 1.4 |
| **Sum g** | | **22.6** | **20.0** | **80.9** | | **10.6** |
| **Sum kJ** | | **384** | **740** | **1376** | **2500** | |
| **% energy** | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | |
| warburtons wholemeal | 155 | 15.8 | 5.4 | 61.4 | 1513 | 9.8 |
| peanut butter whole earth | 55 | 14.3 | 27.6 | 5.6 | 1349 | 4.0 |
| **Sum g** | | **30.1** | **33.0** | **66.9** | | **13.8** |
| **Sum kJ** | | **511** | **1222** | **1138** | **2871** | |
| **% energy** | | **18** | **43** | **40** | **100** | |

## Macaroni Cheese

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| Macaroni, raw | 150 | 18.0 | 2.7 | 111.2 | 2184 | 4.4 |
| Onions, raw | 150 | 1.8 | 0.3 | 11.9 | 225 | 2.1 |
| cheddar full fat | 20 | 5.1 | 7.0 | 0.0 | 345 | |
| cornflour | 50 | 0.3 | 0.4 | 46.0 | 754 | 0.1 |
| butter | 36.4 | 0.2 | 29.9 | 0.2 | 1113 | 0.0 |
| milk, skimmed, | 150 | 5.1 | 0.3 | 6.6 | 204 | 0.0 |
| **Sum g** | | **30.5** | **40.6** | **175.8** | | **6.5** |
| **Sum kJ** | | **518** | **1500** | **2989** | **5008** | |
| **% energy** | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | |
| Macaroni, raw | 165 | 19.8 | 3.0 | 122.3 | 2402 | 4.8 |
| Onions, raw | 50 | 0.6 | 0.1 | 4.0 | 75 | 0.7 |
| Cheddar half fat | 40 | 13.1 | 6.3 | 0.0 | 456 | 0.0 |
| flour white | 31.5 | 3.0 | 0.4 | 24.5 | 457 | 1.0 |
| butter | 37 | 0.2 | 30.4 | 0.2 | 1132 | 0.0 |
| milk, skimmed, | 230 | 7.8 | 0.5 | 10.1 | 313 | 0.0 |
| **Sum g** | | **44.5** | **40.7** | **161.0** | | **6.5** |
| **Sum kJ** | | **756** | **1505** | **2738** | **4999** | |
| **% energy** | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | |
| Macaroni, raw | 128 | 15.4 | 2.3 | 94.9 | 1864 | 3.7 |
| Onions, raw | 50 | 0.6 | 0.1 | 4.0 | 75 | 0.7 |
| Cheddar half fat | 143 | 46.8 | 22.6 | 0.0 | 1632 | 0.0 |
| flour white | 29 | 2.7 | 0.4 | 22.5 | 421 | 0.9 |
| wheat bran | 3 | 0.4 | 0.2 | 0.8 | 26 | 1.1 |
| butter | 18 | 0.1 | 14.8 | 0.1 | 551 | 0.0 |
| milk, skimmed, | 230 | 7.8 | 0.5 | 10.1 | 313 | 0.0 |
| **Sum g** | | **73.8** | **40.8** | **132.4** | | **6.4** |
| **Sum kJ** | | **1255** | **1509** | **2250** | **5014** | |
| **% energy** | | **25** | **30** | **45** | **100** | |

## Vegetable Curry

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| lentils, green and brown, raw | 40 | 9.7 | 0.8 | 19.5 | 506 | 3.6 |
| Onions, raw | 150 | 1.8 | 0.3 | 11.9 | 225 | 2.1 |
| potatoes, new, raw | 590 | 10.0 | 1.8 | 95.0 | 1758 | 5.9 |
| Olive oil | 34 | 0.0 | 34.0 | 0.0 | 1257 | 0.0 |
| Green pepper | 200 | 1.6 | 0.6 | 5.2 | 130 | 0.0 |
| bacon rashers, back, raw | 10 | 1.7 | 1.7 | 0.0 | 7 | 0.0 |
| Carrots | 450 | 2.7 | 1.4 | 35.6 | 657 | 10.8 |
| Tomatoes canned | 300 | 3.0 | 0.3 | 9.0 | 207 | 2.1 |
| **Sum g** | | **30.5** | **40.7** | **176.1** | | **24.5** |
| **Sum kJ** | | **519** | **1506** | **2994** | **5018** | |
| **% energy** | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | |
| lentils, green and brown, raw | 110 | 26.7 | 2.1 | 53.7 | 1390 | 9.8 |
| Onions, raw | 100 | 1.2 | 0.2 | 7.9 | 150 | 1.4 |
| potatoes, new, raw | 350 | 6.0 | 1.1 | 56.4 | 1043 | 3.5 |
| Olive oil | 32 | 0.0 | 32.0 | 0.0 | 1183 | 0.0 |
| Green pepper | 200 | 1.6 | 0.6 | 5.2 | 130 | 0.0 |
| bacon rashers, back, raw | 20 | 3.3 | 3.3 | 0.0 | 13 | 0.0 |
| Carrots | 340 | 2.0 | 1.0 | 26.9 | 496 | 8.2 |
| Tomatoes canned | 400 | 4.0 | 0.4 | 12.0 | 276 | 2.8 |
| **Sum g** | | **44.8** | **40.6** | **162.0** | | **25.7** |
| **Sum kJ** | | **762** | **1503** | **2754** | **5019** | |
| **% energy** | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | |
| lentils, green and brown, raw | 200 | 48.6 | 3.8 | 97.6 | 2528 | 17.8 |
| Onions, raw | 50 | 0.6 | 0.1 | 4.0 | 75 | 0.7 |
| potatoes, new, raw | 90 | 1.5 | 0.3 | 14.5 | 268 | 0.9 |
| Olive oil | 17 | 0.0 | 17.0 | 0.0 | 628 | 0.0 |
| Green pepper | 51 | 0.4 | 0.2 | 1.3 | 33 | 0.0 |
| bacon rashers, back, raw | 110 | 18.2 | 18.2 | 0.0 | 72 | 0.0 |
| Carrots | 85 | 0.5 | 0.3 | 6.7 | 124 | 2.0 |
| Tomatoes canned | 300 | 3.0 | 0.3 | 9.0 | 207 | 2.1 |
| **Sum g** | | **72.8** | **40.0** | **133.1** | | **23.5** |
| **Sum kJ** | | **1238** | **1480** | **2262** | **4980** | |
| **% energy** | | **25** | **30** | **45** | **100** | |

## Raspberry Yoghurt

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| danone | 400 | 14.0 | 11.2 | 46.4 | 1440 | 8.0 |
| sugar, white | 39 | 0.2 | 0.0 | 40.8 | 656 | 0.0 |
| crème fraiche | 23 | 0.5 | 9.2 | 0.6 | 358 | 0.0 |
| **Sum g** | | **14.7** | **20.4** | **87.7** | | **8.0** |
| **Sum kJ** | | **250** | **755** | **1491** | **2496** | |
| **% energy** | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | |
| danone | 440 | 15.4 | 12.3 | 51.0 | 1584 | 8.8 |
| Tofu blue dragon | 90 | 6.2 | 2.4 | 2.2 | 232 | 0.1 |
| sugar, white | 25 | 0.1 | 0.0 | 26.1 | 420 | 0.0 |
| cream, single | 30 | 1.0 | 5.7 | 0.7 | 239 | 0.0 |
| **Sum g** | | **22.7** | **20.5** | **80.0** | | **8.9** |
| **Sum kJ** | | **386** | **758** | **1360** | **2504** | |
| **% energy** | | **15** | **30** | **54** | **100** | |
| **Meal C** | | | | | | |
| danone | 440 | 15.4 | 12.3 | 51.0 | 1584 | 8.8 |
| sugar, white | 8 | 0.0 | 0.0 | 8.4 | 134 | 0.0 |
| Tofu blue dragon | 300 | 20.7 | 8.1 | 7.2 | 774 | 0.3 |
| **Sum g** | | **36.1** | **20.4** | **66.6** | | **9.1** |
| **Sum kJ** | | **614** | **756** | **1132** | **2502** | |
| **% energy** | | **25** | **30** | **45** | **100** | |

## Strawberry Yoghurt

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| danone | 385 | 14.3 | 12.3 | 49.3 | 1536 | 6.2 |
| sugar, white | 37 | 0.2 | 0.0 | 38.7 | 622 | 0.0 |
| crème fraiche | 20 | 0.4 | 8.0 | 0.5 | 311 | 0.0 |
| **Sum g** | | **14.9** | **20.3** | **88.4** | | **6.2** |
| **Sum kJ** | | **253** | **752** | **1503** | **2508** | |
| **% energy** | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | |
| danone | 440 | 16.3 | 14.1 | 56.3 | 1756 | 7.0 |
| Tofu blue dragon | 75 | 5.2 | 2.0 | 1.8 | 194 | 0.1 |
| sugar, white | 21.5 | 0.1 | 0.0 | 22.5 | 361 | 0.0 |
| cream, single | 23 | 0.8 | 4.4 | 0.5 | 184 | 0.0 |
| **Sum g** | | **22.3** | **20.5** | **81.1** | | **19.4** |
| **Sum kJ** | | **379** | **758** | **1379** | **2516** | |
| **% energy** | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | |
| danone | 370 | 13.7 | 11.8 | 47.4 | 1476 | 5.9 |
| sugar, white | 10.5 | 0.1 | 0.0 | 11.0 | 177 | 0.0 |
| Tofu blue dragon | 330 | 22.8 | 8.9 | 7.9 | 851 | 0.3 |
| **Sum g** | | **36.5** | **20.8** | **66.3** | | **6.3** |
| **Sum kJ** | | **621** | **768** | **1126** | **2515** | |
| **% energy** | | **25** | **31** | **45** | **100** | |

## Pasta Bolognaise

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| Pasta shells | 175 | 21.0 | 3.2 | 129.7 | 2548 | 5.1 |
| Olive oil | 31 | 0.0 | 31.0 | 0.0 | 1146 | 0.0 |
| Beef lean raw | 17 | 3.8 | 0.7 | 0.0 | 92 | 0.0 |
| dolmio med veg sauce | 480 | 7.2 | 5.8 | 45.1 | 1118 | 5.3 |
| **Sum g** | | **32.0** | **40.6** | **174.8** | | **10.4** |
| **Sum kJ** | | **544** | **1503** | **2972** | **5019** | |
| **% energy** | | **11** | **30** | **59** | **100** | |
| **Meal B** | | | | | | |
| Pasta shells | 140 | 16.8 | 2.5 | 103.7 | 2038 | 4.1 |
| Olive oil | 27 | 0.0 | 27.0 | 0.0 | 998 | 0.0 |
| Beef lean raw | 80 | 18.0 | 3.4 | 0.0 | 434 | 0.0 |
| dolmio med veg sauce | 620 | 9.3 | 7.4 | 58.3 | 1445 | 6.8 |
| **Sum g** | | **44.1** | **40.4** | **162.0** | | **10.9** |
| **Sum kJ** | | **750** | **1494** | **2754** | **4998** | |
| **% energy** | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | |
| Pasta shells | 103 | 12.4 | 1.9 | 76.3 | 1500 | 3.0 |
| Olive oil | 21 | 0.0 | 21.0 | 0.0 | 776 | 0.0 |
| Beef lean raw | 233 | 52.4 | 10.0 | 0.0 | 1263 | 0.0 |
| dolmio med veg sauce | 600 | 9.0 | 7.2 | 56.4 | 1398 | 6.6 |
| **Sum g** | | **73.8** | **40.1** | **132.7** | | **9.6** |
| **Sum kJ** | | **1254** | **1482** | **2256** | **4993** | |
| **% energy** | | **25** | **30** | **45** | **100** | |

## Chicken and Rice

| | Ingredients (g) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|
| **Meal A** | | | | | | |
| Chicken, light meat raw | 50 | 12.0 | 0.6 | 0.0 | 225 | 0.0 |
| Chicken tonight mushroom | 400 | 3.2 | 21.6 | 25.2 | 1348 | 0.8 |
| Rice basmati,raw | 190 | 14.1 | 1.0 | 151.6 | 2854 | 1.9 |
| Olive oil | 17.5 | 0.0 | 17.5 | 0.0 | 647 | 0.0 |
| **Sum g** | | **29.3** | **40.6** | **176.8** | | **2.7** |
| **Sum kJ** | | **497** | **1502** | **3006** | **5005** | |
| **% energy** | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | |
| Chicken, light meat raw | 120 | 28.8 | 1.3 | 0.0 | 539 | 0.0 |
| Chicken tonight mushroom | 400 | 3.2 | 21.6 | 25.2 | 1348 | 0.8 |
| Rice basmati,raw | 171 | 12.7 | 0.9 | 136.5 | 2568 | 1.7 |
| Olive oil | 16.8 | 0.0 | 16.8 | 0.0 | 621 | 0.0 |
| **Sum g** | | **44.7** | **40.6** | **161.7** | | **2.5** |
| **Sum kJ** | | **759** | **1501** | **2748** | **5008** | |
| **% energy** | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | |
| Chicken, light meat raw | 250 | 60.0 | 2.8 | 0.0 | 1123 | 0.0 |
| Chicken tonight mushroom | 400 | 3.2 | 21.6 | 25.2 | 1348 | 0.8 |
| Rice basmati,raw | 135 | 10.0 | 0.7 | 107.7 | 2028 | 1.4 |
| Olive oil | 15 | 0.0 | 15.0 | 0.0 | 554 | 0.0 |
| **Sum g** | | **73.2** | **40.0** | **132.9** | | **2.2** |
| **Sum kJ** | | **1244** | **1480** | **2260** | **4984** | |
| **% energy** | | **25** | **30** | **45** | **100** | |

## Bread and Butter Pudding

| | Ingredients (g) | Ingredients (number) | Protein (g) | Fat (g) | Carbohydrate (g) | Energy (kJ) | Fibre (g) |
|---|---|---|---|---|---|---|---|
| **Meal A** | | | | | | | |
| Egg whole | 50 | 1 | 6.3 | 5.4 | 0.0 | 313.5 | 0.0 |
| white bread | 55 | | 4.4 | 0.9 | 25.4 | 512.1 | 1.1 |
| raisins | 50 | | 0.6 | 0.3 | 32.1 | 563.5 | 3.4 |
| butter | 12 | | 0.1 | 9.9 | 0.1 | 367.1 | 0.0 |
| sugar, white | 25 | | 0.1 | 0.0 | 26.1 | 420.3 | 0.0 |
| whole milk | 100 | | 3.3 | 3.9 | 4.5 | 274.0 | |
| **Sum g** | | | **14.6** | **20.3** | **88.1** | | **4.5** |
| **Sum kJ** | | | **249** | **751** | **1498** | **2498** | |
| **% energy** | | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | | |
| Egg whole | 100 | 2 | 12.5 | 10.8 | 0.0 | 627.0 | 0.0 |
| white bread | 50 | | 4.0 | 0.8 | 23.1 | 465.5 | 1.0 |
| raisins | 30 | | 0.3 | 0.2 | 19.2 | 338.1 | 2.0 |
| butter | 3.5 | | 0.0 | 2.9 | 0.0 | 107.1 | 0.0 |
| sugar, white | 30 | | 0.2 | 0.0 | 31.4 | 504.3 | 0.0 |
| whole milk | 150 | | 5.0 | 5.9 | 6.8 | 411.0 | |
| **Sum g** | | | **21.9** | **20.5** | **80.4** | | **3.0** |
| **Sum kJ** | | | **372** | **758** | **1367** | **2497** | |
| **% energy** | | | **15** | **30** | **55** | **100** | |
| **Meal C** | | | | | | | |
| Egg whole | 100 | 2 | 12.5 | 10.8 | 0.0 | 627.0 | 0.0 |
| Egg white | 128 | 4 | 11.5 | 0.0 | 0.0 | 195.8 | 0.0 |
| white bread | 50 | | 4.0 | 0.8 | 23.1 | 465.5 | 1.0 |
| raisins | 22 | | 0.2 | 0.1 | 14.1 | 247.9 | 1.5 |
| Almonds | 10 | | 2.1 | 5.6 | 0.7 | 253.4 | 0.7 |
| butter | 3.5 | | 0.0 | 2.9 | 0.0 | 107.1 | 0.0 |
| sugar, white | 20 | | 0.1 | 0.0 | 20.9 | 336.2 | 0.0 |
| milk, skimmed, | 175 | 159.08 | 6.0 | 0.4 | 7.7 | 238.0 | 0.0 |
| **Sum g** | | | **36.4** | **20.5** | **66.5** | | **3.2** |
| **Sum kJ** | | | **619** | **759** | **1130** | **2508** | |
| **% energy** | | | **25** | **30** | **45** | **100** | |

| **Rice Pudding** | | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Ingredients (g)** | **Ingredients (number)** | **Protein (g)** | **Fat (g)** | **Carbohydrate (g)** | **Energy (kJ)** | **Fibre (g)** |
| **Meal A** | | | | | | | |
| flaked pudding rice | 60 | | 0.1 | 0.5 | 51.3 | 891 | 0.4 |
| Egg whole | 50 | 1 | 6.3 | 5.4 | 0.0 | 314 | 0.0 |
| Egg white | 32 | 1 | 2.9 | 0.0 | 0.0 | 49 | 0.0 |
| butter | 10 | | 0.1 | 8.2 | 0.1 | 306 | 0.0 |
| sugar, white | 30 | | 0.2 | 0.0 | 31.4 | 504 | 0.0 |
| milk, skimmed, | 120 | | 4.1 | 0.2 | 5.3 | 163 | 0.0 |
| cream, single | 31 | | 1.0 | 5.9 | 0.7 | 247 | |
| **Sum g** | | | **14.5** | **20.3** | **88.7** | | **0.4** |
| **Sum kJ** | | | **247** | **750** | **1507** | **2504** | |
| **% energy** | | | **10** | **30** | **60** | **100** | |
| **Meal B** | | | | | | | |
| flaked pudding rice | 55 | | 0.1 | 0.4 | 47.0 | 817 | 0.4 |
| Egg whole | 150 | 3 | 18.8 | 16.2 | 0.0 | 941 | 0.0 |
| sugar, white | 30 | | 0.2 | 0.0 | 31.4 | 504 | 0.0 |
| milk, skimmed, | 50 | | 1.7 | 0.1 | 2.2 | 68 | 0.0 |
| cream, single | 20 | | 0.7 | 3.8 | 0.4 | 160 | |
| **Sum g** | | | **21.3** | **20.6** | **81.0** | | **0.4** |
| **Sum kJ** | | | **362** | **761** | **1377** | **2500** | |
| **% energy** (from H) | | | **14** | **30** | **55** | **100** | |
| **Meal C** | | | | | | | |
| flaked pudding rice | 45 | | 0.1 | 0.4 | 38.5 | 668 | 0.3 |
| Egg whole | 150 | 3 | 18.8 | 16.2 | 0.0 | 941 | 0.0 |
| Egg white | 128 | 4 | 11.5 | 0.0 | 0.0 | 196 | 0.0 |
| sugar, white | 20 | | 0.1 | 0.0 | 20.9 | 336 | 0.0 |
| milk, skimmed, | 150 | | 5.1 | 0.3 | 6.6 | 204 | 0.0 |
| cream, single | 20 | | 0.7 | 3.8 | 0.4 | 160 | |
| **Sum g** | | | **36.2** | **20.7** | **66.4** | | **0.3** |
| **Sum kJ** | | | **615** | **765** | **1129** | **2509** | |
| **% energy** | | | **24.5** | **30.5** | **45.0** | **100** | |

You are invited to take part in a research study. Before you decide if you wish to take part it is important that you understand what the research will involve. Please take as much time as you would like to read this information carefully.

**What is the purpose of this study?**
Weight control is a balance between the food we eat and the amount of energy we produce (at rest or with exercise). The effect of different foods on energy balance is unclear. In this study we will see how different types of food affect energy balance and some of the hormones and metabolic factors that are produced when we eat.

**Who will be involved?**
We need healthy men or women, 18 to 70 years, normal weight or overweight, to take part in this study. You need to be a non-smoker, not pregnant, and able to eat all kinds of food.

**Do I have to take part?**
It is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form. However, you will still be free to withdraw at any time and without giving a reason.

**What do I have to do?**
On three occasions you will spend five days with us so that we can accurately measure your energy balance. You will spend the nights at the Clinical Research Facility (CRF) at Addenbrooke's Hospital and most of the days at MRC HNR. You will be provided with all of your food. During the day you will be able to read, relax or work. We can provide power points, a computer with internet access, television and a comfortable lounge. You will go for a walk each afternoon. Each visit will be three weeks apart and you will not need to change any aspects of your life in between visits. We will ask you not to donate blood during the study.

**What will happen to me if I take part?**
Your first visit will last about 30 minutes. You can ask us any questions and we can see if the study might suit you. We will take some measurements and ask you to answer a brief questionnaire. If you are overweight we will ask you to drink a glucose drink and we will measure your blood glucose after 2 hours.
You will come to the CRF in the evening. Before Day 1 we will ask you to collect your urine for 24 hours and complete a record of the food you eat over 4 days.
Day 1 Before breakfast we will take a blood sample (10mls – about 2 teaspoons), and you will have a breathing test and some tests to measure your body fat:
- Dual energy X-Ray absorptiometry (DXA) scan uses a small amount of X-rays and requires you to sit or lie quietly for about 10 minutes.
- ECHO MRI uses a magnet to produce an image. It does not use radiation. You will be asked to lie flat on a bed which passes into the scanner. You cannot have this scan if you have any metal in your body and you may not want to have it if you experience claustrophobia.
-Bod Pod measures body volume while you sit in a small enclosed chamber wearing a swimsuit for 5 min.
You will be transported to MRC HNR where you will have all your meals. There will be plenty of food available and you can ask for meals or snacks whenever you wish. You will return to the CRF in the evening with some snacks.
Day 2 Will be spent at MRC HNR. At the end of the day we will attach a small monitor, similar to a pager, so that we can frequently measure your blood sugar. A very small needle is placed under your skin and the monitor is taped down so that it won't interfere with any activities and you can wear it for the next two days.
Day 3 You will have your meals at MRC HNR and when you return to the CRF you will move in to the room calorimeter. This is a comfortable living room where we can measure your energy expenditure, and how much fat, carbohydrate and protein your body uses. Measurements are made continuously, so we would like you to remain in the room for 36 hours.
Day 4 You will be in the room calorimeter. We will provide you with a set amount of food and will take a number of blood tests from a cannula between breakfast and lunch. This is a small plastic tube inserted into your vein with a needle. The needle is removed and we can take samples without requiring any other needles. 8 samples will be taken in total (about 120mls – half a cup). We will ask you to collect all your urine while you are in the calorimeter. Between tests you can work or relax as you wish and will have access to the internet, TV, DVD player etc. You will spend some time in the afternoon on an exercise cycle.
Day 5 You will have the same tests as day 1 and you will be able to go home after breakfast.

**What are the possible benefits?**
You will be screened for diabetes, high blood pressure, and osteoporosis. You (and your GP, with consent) will receive results of all clinically relevant investigations.

**What are the possible disadvantages and risks?**

There is a risk of bruising and brief discomfort when you have a blood test or a cannula inserted.

DXA will expose you to a very small amount of X-ray radiation. For the whole study this is equivalent to less than 1 day of the background radiation we receive in Cambridge every day.
If a new diagnosis of diabetes, high blood pressure or osteoporosis is made this could affect your future insurance status (e.g. for life insurance or private medical insurance).

**What will happen if anything goes wrong?**
If something goes wrong any procedures will be stopped and you will be seen by one of the clinicians. Your involvement in the rest of the study may be stopped.
If you have any other problems, illnesses or concerns during the study you should discuss these with the investigator.
Any complaints you have about this study will be fully investigated. For research carried out at MRC HNR participants would be in the same position as if public liability insurance had been taken out. If you wish to make a complaint you can contact our Unit Manager.

**Will my taking part in this study be kept confidential?**
Any information that is collected about you during the course of the research will be kept strictly confidential. Your GP will be notified that you are participating in this study.

**What will happen to the study results?**
We will inform you of your study results and with your permission your GP will be notified. As the samples from the study will not be processed until the end of the study, your results will not be available for 12 months. The overall results may be presented at scientific meetings or published in a scientific journal. You will not be identified in any of these presentations or publications. A summary of the study results will be available on MRC HNR website. We will be happy to discuss the results with you when the study is completed, and will let you know where you can obtain a copy of the published results.

**Will I be reimbursed for my time?**
In recognition of your time commitment, you will be paid an honorarium of £200 for the study. Reasonable travel expenses will also be paid.

**Who is organising and funding the study?**
This study is being organised and funded by the Nutrition and Health section at MRC HNR and the Wellcome Trust Clinical Research Facility.

**Who has reviewed the study?**
This study has been reviewed by the Scientific Co-ordination Committee of MRC HNR, the Scientific Advisory board of the CRF, Addenbrooke's NHS Trust Research and Development committee and by Cambridge Local Research Ethics Committee.

**Contact for further information**

If you have any further questions please contact Dr Rosemary Hall at MRC HNR on 01223 426356 or rosemary.hall@mrc-hnr.cam.ac.uk.

**And finally...**

Thank you for having taken the time to read this information sheet and your interest in the study. If you do decide to take part in the study, you will be given a copy of this information sheet and your signed consent form for you to keep.

MRC Human Nutrition Research

# *Diet and Energy Metabolism*

## Information Sheet for Study Participants

Principal Investigator: Dr Rosemary Hall

MRC Human Nutrition Research
Elsie Widdowson Laboratory
Fulbourn Road
Cambridge
CB1 9NL

Telephone: 01223 426356
Fax: 01223 437515
research@mrc-hnr.cam.ac.uk
http://www.mrc-hnr.cam.ac.uk

Version 1
12 April 2006

MRC | Human Nutrition Research

# Diet and Energy Metabolism - Consent Form

LREC Reference Number: 06/Q0108/181
Name of Principal Investigator: Dr Rosemary Hall

**Please initial box**

1. I confirm that I have read and understand the information sheet dated 12 April 2006 (version 01) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, and without my medical care or legal rights being affected. ☐
3. I consent to my general practitioner being notified of my participation in this research. ☐
4. I consent to my general practitioner being notified of my study results and any abnormalities found during this research. ☐
5. I understand that samples taken as part of the protocol of this study may be stored for up to 10 years and used in further research studies. Samples will only be used in studies that have been approved by the appropriate Ethics Committee. ☐
6. I understand that samples taken as part of this study may be analysed in another laboratory outside of MRC Human Nutrition Research. All samples will be made anonymous, and no personal information will be sent to another laboratory. ☐
7. I have read the DXA Information Leaflet and will inform the research team if I am pregnant. ☐
8. I understand that I cannot participate in this research if I am pregnant. I am not pregnant and will inform the research team if I become pregnant. ☐
9. I agree to take part in the above study. ☐

| Name of Volunteer *(Please print)* | Date | Signature |
|---|---|---|
| Name of Research Team Member *(Please print)* | Date | Signature |
| Name of Researcher *(Please print)* | Date | Signature |

***3 copies required:*** top copy for researcher; one copy for volunteer; one copy to be kept with research subject's notes.

# Diet and Energy Metabolism

## Telephone Screening Questionnaire

Name .....

Address .....

.....

Age ..... Date of Birth .....

Phone Number: Day ..... Evening .....

Best time to phone .....

General Practitioner .....

Of (medical practice).....

GP telephone number.....

How did you hear about the study? (Advertisement/Database/etc).....
Are you currently in any other research studies, or have you been in any in the last year?
.....

**Health**

Current weight _____kg Height _____m Estimated BMI _____ $kg/m^2$

Do you have or have you had any of the following conditions?

| | YES | NO |
|---|---|---|
| (x) Diabetes | ☐ | ☐ |
| (xi) High Blood Pressure | ☐ | ☐ |
| (xii) Stomach or bowel problems | ☐ | ☐ |
| (xiii) Asthma/Eczema/Hayfever | ☐ | ☐ |
| (xiv) High Cholesterol | ☐ | ☐ |
| (xv) Chronic medical conditions (heart, kidney, liver, thyroid) | ☐ | ☐ |
| (xvi) Anaemia | ☐ | ☐ |
| (xvii) Food/Drug allergies | ☐ | ☐ |
| (xviii) Eating Disorder | ☐ | ☐ |
| (xix) Are you Pregnant or Breastfeeding (or planning pregnancy) | ☐ | ☐ |
| (xx) Do you have a pacemaker or metal implants | ☐ | ☐ |

Please list any others .....
.....
.....
.....

Please list any medications (pills, tablets, contraceptives, inhalers etc.) that you are taking, either prescribed by your doctor or purchased over the counter.
.....
.....
.....

| | YES | NO |
|---|---|---|
| Do you smoke?<br>If yes, how many cigarettes per day? ..... | ☐ | ☐ |
| Do you drink alcohol?<br>If yes, how many units of alcohol would you consume in an average week?<br>(1 unit = 1 measure of spirits / 1 small glass of wine / 1 half pint of beer)<br>..... | ☐ | ☐ |

**Availability and Transport**

| | YES | NO |
|---|---|---|
| Would you have any difficulty in: | | |
| 6. Living in for five days on three occasions three weeks apart? | ☐ | ☐ |
| 7. Eating all types of food? | ☐ | ☐ |
| 8. Having a blood sample taken? | ☐ | ☐ |
| 9. Spending time in a room calorimeter? | ☐ | ☐ |
| 10. Having a DXA, MRI, BOD POD measurement? | ☐ | ☐ |
| Would you have any anticipated difficulties with your availability or transport arrangements to attend each of these sessions? | ☐ | ☐ |
| Do you have any preferred days to attend the unit?<br>If yes, please give days.<br>..... | ☐ | ☐ |

_____ _____ _____

Name of Research Team member (*Please print*) Date Signature

Eligible for this study YES NO

If YES: Send Information Sheet..... YES NO
Next Appointment.....
.....

If NO: Would you like information about other studies at MRC HNR or consider placing your name on our Volunteer Database? YES NO
Questionnaire Sent..... YES NO

**Address**

**Date**

Dear

**Re: Diet and Energy Metabolism**

We are currently recruiting volunteers for a nutrition study at MRC Human Nutrition Research. We have looked at the information that we have on our volunteer database and found that you may be eligible to be involved.

This study will investigate the effect of different diets on energy balance.

We are looking for a total of 20 volunteers for this study. Each volunteer will be required to spend five days (and nights) at MRC Human Nutrition Research and the Wellcome Trust Clinical Research Facility on three occasions. We will provide you with all of your food and measure the effects of different diets on the hormones and metabolic factors produced after eating, your energy expenditure, and your body composition. In between tests you will be able to relax, read, work, access the internet, watch TV, etc and will have a period of exercise each day.

In recognition of your time commitment, you will receive an honorarium of £200. Reasonable travel expenses will also be reimbursed.

Please read the enclosed volunteer information sheet for more details, and if you are interested in taking part, or if you have any questions, please contact me on 01223 426356.

Kind regards

Dr Rosemary Hall
Clinical Scientist

Dr. .....
*GP Address*

*Date*

Dear Dr .....,

**Re: Diet and Energy Metabolism**

Your patient *Name, DOB & Address of Volunteer,* has volunteered to take part in a research study at MRC Human Nutrition Research (HNR). *He/She* has given us permission to contact you.

The study is designed to investigate the effect of different diets on energy intake and expenditure. On three occasions participants will be required to spend five days at MRC HNR and the Welcome Trust Clinical Research Facility, Addenbrooke's Hospital. They will be provided with all their food and we will measure metabolic factors and satiety hormones associated with appetite and the postprandial response to eating, energy expenditure and body composition. Part of this time will be spent in a room calorimeter. (For more information see the attached volunteer information sheet). We will have two groups – lean (BMI <25) and obese (BMI 30-40kg/m2)

Your patient has been asked to provide us with their medical history. Volunteers will be excluded if they are smokers, are pregnant or planning pregnancy, have any known chronic disease, are restrained eaters, or are unable to eat any of the study foods.

We will inform you if any abnormal results are obtained which may be of clinical significance.

**If you feel that there is a medical reason which should prevent their participation in this study or if you need further details please contact our clinician on 01223 - 426356.**

Yours sincerely

Dr Rosemary Hall
Clinical Scientist

# Volunteers Wanted
## For Nutrition Research

We are running a study to investigate how different diets affect energy balance.

---

Are you:

- Healthy
- Aged 18-70 years
- Willing to spend 5 days and nights living at MRC HNR and Wellcome Trust Clinical Research Facility on 3 occasions
- Able to eat all kinds of food

---

**If you would like more information please contact Rosemary Hall on (01223) 426356**
**rosemary.hall@mrc-hnr.cam.ac.uk**

You will be reimbursed for participation in the study and for reasonable travel expenses

Version 01 14/04/06

## APPENDIX: THREE-FACTOR EATING QUESTIONNAIRE

One point is given for each item in Part I and for each item (numbered question) in Part II. The correct answer for the true/false items is underlined and beside it is the number of the factor that it measures. The direction of the question in Part II is determined by splitting the responses at the middle. If the item is labelled '+', those responses above the middle are given a zero. Vice versa for those with a '−'. For example, anyone scoring 3 or 4 on the first item in Part II (item No. 37) would receive one point. Anyone scoring 1 or 2 would receive a zero.

### Part I

| | | | | Factor Number |
|---|---|---|---|---|
| 1. | When I smell a sizzling steak or see a juicy piece of meat, I find it very difficult to keep from eating, even if I have just finished a meal. | T | F | 2 |
| 2. | I usually eat too much at social occasions, like parties and picnics. | T | F | 2 |
| 3. | I am usually so hungry that I eat more than three times a day. | T | F | 3 |
| 4. | When I have eaten my quota of calories, I am usually good about not eating any more. | T | F | 1 |
| 5. | Dieting is so hard for me because I just get too hungry. | T | F | 3 |
| 6. | I deliberately take small helpings as a means of controlling my weight. | T | F | 1 |
| 7. | Sometimes things just taste so good that I keep on eating even when I am no longer hungry. | T | F | 2 |
| 8. | Since I am often hungry, I sometimes wish that while I am eating, an expert would tell me that I have had enough or that I can have something more to eat. | T | F | 3 |
| 9. | When I feel anxious, I find myself eating. | T | F | 2 |
| 10. | Life is too short to worry about dieting. | T | F | 1 |
| 11. | Since my weight goes up and down, I have gone on reducing diets more than once. | T | F | 2 |
| 12. | I often feel so hungry that I just have to eat something. | T | F | 3 |
| 13. | When I am with someone who is overeating, I usually overeat too. | T | F | 2 |
| 14. | I have a pretty good idea of the number of calories in common food. | T | F | 1 |
| 15. | Sometimes when I start eating, I just can't seem to stop. | T | F | 2 |
| 16. | It is not difficult for me to leave something on my plate. | T | F | 2 |
| 17. | At certain times of the day, I get hungry because I have gotten used to eating then. | T | F | 3 |
| 18. | While on a diet, if I eat food that is not allowed, I consciously eat less for a period of time to make up for it. | T | F | 1 |
| 19. | Being with someone who is eating often makes me hungry enough to eat also. | T | F | 3 |
| 20. | When I feel blue, I often overeat. | T | F | 2 |
| 21. | I enjoy eating too much to spoil it by counting calories or watching my weight. | T | F | 1 |
| 22. | When I see a real delicacy, I often get so hungry that I have to eat right away. | T | F | 3 |
| 23. | I often stop eating when I am not really full as a conscious means of limiting the amount that I eat. | T | F | 1 |
| 24. | I get so hungry that my stomach often seems like a bottomless pit. | T | F | 3 |
| 25. | My weight has hardly changed at all in the last ten years. | T | F | 2 |
| 26. | I am always hungry so it is hard for me to stop eating before I finish the food on my plate. | T | F | 3 |
| 27. | When I feel lonely, I console myself by eating. | T | F | 2 |
| 28. | I consciously hold back at meals in order not to gain weight. | T | F | 1 |
| 29. | I sometimes get very hungry late in the evening or at night. | T | F | 3 |

| | | | | |
|---|---|---|---|---|
| 30. | I eat anything I want, any time I want. | T | F | 1 |
| 31. | Without even thinking about it, I take a long time to eat. | T | F | 2 |
| 32. | I count calories as a conscious means of controlling my weight. | T | F | 1 |
| 33. | I do not eat some foods because they make me fat. | T | F | 1 |
| 34. | I am always hungry enough to eat at any time. | T | F | 3 |
| 35. | I pay a great deal of attention to changes in my figure. | T | F | 1 |
| 36. | While on a diet, if I eat a food that is not allowed, I often then splurge and eat other high calorie foods. | T | F | 2 |

## Part II

Directions: Please answer the following questions by circling the number above the response that is appropriate to you.

37. How often are you dieting in a conscious effort to control your weight?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| rarely | sometimes | usually | always | + 1 |

38. Would a weight fluctuation of 5 lbs affect the way you live your life?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| not at all | slightly | moderately | very much | + 1 |

39. How often do you feel hungry?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| only at mealtimes | sometimes between meals | often between meals | almost always | + 3 |

40. Do your feelings of guilt about overeating help you to control your food intake?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| never | rarely | often | always | + 1 |

41. How difficult would it be for you to stop eating halfway through dinner and not eat for the next four hours?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| easy | slightly difficult | moderately difficult | very difficult | + 3 |

42. How conscious are you of what you are eating?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| not at all | slightly | moderately | extremely | + 1 |

43. How frequently do you avoid 'stocking up' on tempting foods?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| almost never | seldom | usually | almost always | + 1 |

44. How likely are you to shop for low calorie foods?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| unlikely | slightly unlikely | moderately likely | very likely | + 1 |

45. Do you eat sensibly in front of others and splurge alone?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| never | rarely | often | always | + 2 |

46. How likely are you to consciously eat slowly in order to cut down on how much you eat?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| unlikely | slightly likely | moderately likely | very likely | + 1 |

47. How frequently do you skip dessert because you are no longer hungry?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| almost never | seldom | at least once a week | almost every day | − 3 |

48. How likely are you to consciously eat less than you want?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| unlikely | slightly likely | moderately likely | very likely | + 1 |

49. Do you go on eating binges though you are not hungry?

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| never | rarely | sometimes | at least once a week | + 2 |

50. On a scale of 0 to 5, where 0 means no restraint in eating (eating whatever you want, whenever you want it) and 5 means total restraint (constantly limiting food intake and never 'giving in'), what number would you give yourself?

0
eat whatever you want, whenever you want it + 1

1
usually eat whatever you want, whenever you want it

2
often eat whatever you want, whenever you want it

3
often limit food intake, but often 'give in'

4
usually limit food intake, rarely 'give in'

5
constantly limiting food intake, never 'giving in'

51. To what extent does this statement describe your eating behavior? 'I start dieting in the morning, but because of any number of things that happen during the day, by evening I have given up and eat what I want, promising myself to start dieting again tomorrow.'

| 1 | 2 | 3 | 4 | |
|---|---|---|---|---|
| not like me | little like me | pretty good description of me | describes me perfectly | + 2 |

**Diet and Energy Metabolism**

**Visit 1 Questionnaire (EAT-26)**

Date ☐☐/☐☐/☐☐

Participant ID ☐☐☐☐☐☐☐

**Please respond to how each of the following statements apply to you:**

| | Always | Usually | Often | Sometimes | Rarely | Never | Score |
|---|---|---|---|---|---|---|---|
| 1. Am terrified about being overweight | O | O | O | O | O | O | ___ |
| 2. Avoid eating when I am hungry | O | O | O | O | O | O | ___ |
| 3. Find myself preoccupied with food | O | O | O | O | O | O | ___ |
| 4. Have gone on eating binges where I feel that I may not be able to stop | O | O | O | O | O | O | ___ |
| 5. Cut my food into small pieces | O | O | O | O | O | O | ___ |
| 6. Aware of the calorie content of foods that I eat | O | O | O | O | O | O | ___ |
| 7. Particularly avoid food with a high carbohydrate content (i.e. bread, rice, potatoes, etc.) | O | O | O | O | O | O | ___ |
| 8. Feel that others would prefer if I ate more | O | O | O | O | O | O | ___ |
| 9. Vomit after I have eaten | O | O | O | O | O | O | ___ |
| 10. Feel extremely guilty after eating | O | O | O | O | O | O | ___ |
| 11. Am preoccupied with a desire to be thinner | O | O | O | O | O | O | ___ |
| 12. Think about burning up calories when I exercise | O | O | O | O | O | O | ___ |
| 13. Other people think that I am too thin | O | O | O | O | O | O | ___ |
| 14. Am preoccupied with the thought of having fat on my body | O | O | O | O | O | O | ___ |
| 15. Take longer than others to eat my meals | O | O | O | O | O | O | ___ |
| 16. Avoid foods with sugar in them | O | O | O | O | O | O | ___ |
| 17. Eat diet foods | O | O | O | O | O | O | ___ |
| 18. Feel that food controls my life | O | O | O | O | O | O | ___ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 19. Display self-control around food | O | O | O | O | O | O | ___ |
| 20. Feel that others pressure me to eat | O | O | O | O | O | O | ___ |
| 21. Give too much time and thought to food | O | O | O | O | O | O | ___ |
| 22. Feel uncomfortable after eating sweets | O | O | O | O | O | O | ___ |
| 23. Engage in dieting behavior | O | O | O | O | O | O | ___ |
| 24. Like my stomach to be empty | O | O | O | O | O | O | ___ |
| 25. Enjoy trying new rich foods | O | O | O | O | O | O | ___ |
| 26. Have the impulse to vomit after meals | O | O | O | O | O | O | ___ |
| Total Score (see below for scoring instructions) | | | | | | | ___ |

Please respond to each of the following questions:

1) Have you gone on eating binges where you feel that you may not be able to stop? (Eating much more than most people would eat under the same circumstances)

No O Yes O ⇨How many times in the last 6 months? _____

2) Have you ever made yourself sick (vomited) to control your weight or shape?

No O Yes O ⇨How many times in the last 6 months? _____

3) Have you ever used laxatives, diet pills or diuretics (water pills) to control your weight or shape? No O Yes O ⇨How many times in the last 6 months? _____

4) Have you ever been treated for an eating disorder?

No O Yes O ⇨When? _____

5) Have you recently thought of or attempted suicide?

No O Yes O ⇨When? _____

**Scoring System**

| | **Always** | **Usually** | **Often** | **Sometimes** | **Rarely** | **Never** |
|---|---|---|---|---|---|---|
| All questions except 25. | 3 | 2 | 1 | 0 | 0 | 0 |
| Question 25 | 0 | 0 | 0 | 1 | 2 | 3 |

Scores above 20 require further investigation.

Further investigation is also required if the response to one of the final 5 behavioural questions is 'Yes'.

# INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.
Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

## *PART 1: JOB-RELATED PHYSICAL ACTIVITY*

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?
Yes
No

***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**?

Think about only those physical activities that you did for at least 10 minutes at a time.

_____ **days per week**
No vigorous job-related physical activity ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

_____ **days per week**
No moderate job-related physical activity ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**
No job-related walking ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

***PART 2: TRANSPORTATION PHYSICAL ACTIVITY***

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.
8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**
No traveling in a motor vehicle ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**
No bicycling from place to place ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**
No walking from place to place ***Skip to PART 3: HOUSEWORK,***

***HOUSE MAINTENANCE, AND***
***CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

***PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard**?

_____ **days per week**
No vigorous activity in garden or yard ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard**?

_____ **days per week**
No moderate activity in garden or yard ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**
No moderate activity inside home ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

***PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY***

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how

many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**
No walking in leisure time ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**
No vigorous activity in leisure time ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**?

_____ **days per week**
No moderate activity in leisure time ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

***PART 5: TIME SPENT SITTING***

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

_____ **hours per day**
_____ **minutes per day**

## This is the end of the questionnaire, thank you for participating.

MRC | Human Nutrition Research

# WEARING THE 24-HOUR BLOOD GLUCOSE MONITOR

If you experience any problems or have any questions at any time whilst wearing the monitor, please contact:

Rosemary on
01223 426356
(07738 597193 out of hours)

or

Medtronic on 01923212213

# ENTERING BLOOD GLUCOSE VALUES

You must enter at least 4 blood glucose readings into the monitor each day. Blood glucose readings can be taken at any time, but avoid taking them immediately following food or exercise.

To take a fingerstick glucose reading:

- Insert a test strip into the fingerstick meter, with the yellow window facing up – the meter will turn on automatically.
- When the blood drop symbol flashes the meter is ready.
- Obtain a blood sample using the lancet – press the end of the 'pen' so that the button on the side becomes yellow, and then hold the end of the 'pen' firmly against the side of your finger. Press the yellow button to perform the finger prick.
- Obtain a small drop of blood on your finger, and then touch and hold this drop of blood to the edge of the yellow window on the test strip, within the curve. Hold your finger to the edge of the strip until the yellow window is completely filled with blood.
- The blood will be drawn automatically into the strip, and the meter will beep when the test is beginning, a box will then rotate on the screen until the measurement is completed.
- The result will then appear on the screen.

This result should be entered into the blood glucose monitor within 5 minutes. To do this:

- Press **SEL** once – "METER BG" will show
- Press **ACT** once – the monitor screen will then change to "---" and "ENTER BG"
- Use the **UP arrow** to enter your glucose reading, and then press **ACT** to enter this.

If an incorrect glucose meter value is entered into the monitor, it cannot be removed – don't worry! Just enter the correct value within 5 minutes

# RECORDING EVENTS

**Every time you eat or take exercise, please record this in the monitor.**

To do this:

- From the home screen, press **SEL** twice – "EVENT" will show
- Press **ACT** once – the display will flash
- Use the UP arrow to scroll through the options until you reach the appropriate one – "FOOD", "MEDS", "EXERCISE" or "OTHER"
- Press **ACT** to enter the appropriate event
- You are most likely to use only "FOOD" and "EXERCISE". Enter "MEDS" if you take any medication, and "OTHER" for any other event you feel may be important. Please make a note detailing any exercise, medication or other event on the log sheet provided (see below for example). Details of meals or snacks will be recorded in your food diary.

| EVENT | DETAIL |
|---|---|
| Exercise | 30 minutes walking the dog |
| Other | Felt unwell with a migraine |
| | |

# ALARMS

This is unlikely to happen, but the monitor may alarm. The most likely reasons for this are that the sensor has become disconnected or that a calibration error has occurred.

## Sensor Disconnected

This can occur if the sensor becomes disconnected from the monitor, or if the sensor is pulled out of the insertion site.

The alarm will beep every 15 seconds and "DISCONN-" will appear on the screen.

- Turn off the alarm by pressing **SEL** and then **ACT**
- Check the sensor-to-cable and cable-to-monitor connections, to make sure they are tight.
- Make sure the sensor is inserted and secure. If it has come out, don't worry or try to reinsert it, just contact Rosemary.
- If the glucose monitor has been disconnected, reconnect it, wait 30 minutes, and then recalibrate the monitor with a fingerstick blood glucose reading.

## Calibration error

This can occur if the blood glucose value entered into the monitor does not agree with that being recorded, or if an initial blood glucose reading is not entered into the monitor.

The alarm will beep every 15 seconds and "CAL ERROR" will appear on the screen.

- Turn off the alarm by pressing **SEL** and then **ACT**. The screen will change back to the home screen.
- Press **SEL** once to go to the "METER BG" screen – check to make sure that the most recently entered blood glucose value is correct.

If an incorrect value was entered by mistake, take another blood glucose reading and enter the correct value as soon as possible.